

**Molecular Mechanisms of Drug
Resistance in K562 Multidrug Resistant
Leukaemic Cell Lines**

Jodie Stephenson

PhD

2005

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Candidate

A handwritten signature in dark ink, appearing to read "J. Stepan", is written over a horizontal line.

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TABLE OF CONTENTS

| | |
|--|-----------|
| Certificate of authentication | i |
| Acknowledgements | ii |
| List of figures and tables | vii |
| Abbreviations | x |
| Abstract | xiii |
| | |
| CHAPTER 1 Introduction | 1 |
| 1.1 Cancer | 1 |
| 1.2 The Problem of Multidrug Resistance in Cancer | 2 |
| 1.3 P-glycoprotein Mediated MDR | 5 |
| 1.3.1 P-glycoprotein expression in normal tissues | 9 |
| 1.3.2 P-glycoprotein expression in cancer <i>in vivo</i> and <i>in vitro</i> | 10 |
| 1.4 Mechanisms for the activation of P-gp | 13 |
| 1.4.1 Methylation | 13 |
| 1.4.2 Gene rearrangement | 15 |
| 1.4.3 Gene amplification | 16 |
| 1.4.4 Transcriptional activation | 16 |
| 1.4.5 Post transcriptional mechanisms | 25 |
| 1.5 The current study | 26 |
| | |
| CHAPTER 2 Characterisation of experimental cell lines | 28 |
| 2.1 Introduction | 28 |
| 2.2 Materials and Methods | 29 |
| 2.2.1 Cell lines and culture | 29 |
| 2.2.2 Cytotoxicity assays | 29 |
| 2.2.3 Total RNA extraction | 29 |

| | | |
|------------------|---|-----------|
| 2.2.4 | RNA transfer and Northern hybridisation | 31 |
| 2.2.5 | Isolation of genomic DNA | 32 |
| 2.2.6 | Slot Blot Transfer and Southern hybridisation | 33 |
| 2.2.7 | Drug induction | 34 |
| 2.2.7.1 | 3 day treatments | 34 |
| 2.2.7.2 | 4 and 16 hour drug induction treatments | 34 |
| 2.2.8 | Trichostatin A treatment of cells | 35 |
| 2.3 | Results | 35 |
| 2.3.1 | Drug Resistance levels of K562 MDR cell lines | 35 |
| 2.3.2 | <i>MDR1</i> mRNA levels in K562 and MDR cell lines | 37 |
| 2.3.3 | <i>MDR1</i> gene copy number | 40 |
| 2.3.4 | Stability of MDR in K562 MDR sublines | 40 |
| 2.3.5 | Effect of 3 day re-exposure to drug | 44 |
| 2.3.6 | <i>MDR1</i> mRNA Levels in re-stimulated K562 sublines | 51 |
| 2.3.7 | Induction by short term exposure to <i>MDR1</i> substrate drugs | 51 |
| 2.3.8 | Induction by short term exposure to cisplatin | 54 |
| 2.3.9 | Induction by short term exposure to rifampicin | 61 |
| 2.3.10 | Effect of short term exposure to the histone deacetylase inhibitor TSA | 61 |
| 2.4 | Summary of Results | 63 |
| CHAPTER 3 | Investigation of binding to transcription factor sites in the <i>MDR1</i> promoter | 68 |
| 3.1 | Introduction | 68 |
| 3.2 | Materials and Methods | 70 |
| 3.2.1 | EMSAs | 70 |
| 3.2.1.1 | Nuclear extract preparation | 70 |
| 3.2.1.2 | Preparation of labelled binding DNA | 71 |
| 3.2.1.3 | Binding reactions | 73 |
| 3.2.2 | Transient transfections | 73 |

| | | |
|------------------|---|------------|
| 3.2.2.1 | Reporter assays | 74 |
| 3.2.2.2 | Statistical analysis | 75 |
| 3.3 | Results | 75 |
| 3.3.1 | Preparation of nuclear extracts | 75 |
| 3.3.2 | Y-box binding factor levels in K562 and MDR sublines | 76 |
| 3.3.3 | Identification of Y-box binding factor | 79 |
| 3.3.4 | -55GC box binding factor levels in K562 and MDR sublines | 81 |
| 3.3.5 | Transient transfection of <i>MDR1</i> promoter | 85 |
| 3.3.6 | Repressor Binding Protein levels in K562 and K/EPR MDR cell lines | 88 |
| 3.4 | Summary of Results | 91 |
| CHAPTER 4 | <i>MDR1</i> Promoter Methylation Studies | 93 |
| 4.1 | Introduction | 93 |
| 4.2 | Materials and Methods | 94 |
| 4.2.1 | Genomic sequencing | 94 |
| 4.2.1.1 | Bisulphite conversion of genomic DNA | 94 |
| 4.2.1.2 | PCR of converted genomic DNA | 95 |
| 4.2.1.3 | Subcloning of PCR products | 95 |
| 4.3 | Results | 98 |
| 4.3.1 | Methylation status in K562 and K/EPR cells | 98 |
| 4.3.2 | Protein binding levels at CpG sites 43/44 | 98 |
| 4.4 | Summary of Results | 101 |
| CHAPTER 5 | <i>MDR1</i> mRNA Stability Studies | 105 |
| 5.1 | Introduction | 105 |
| 5.2 | Materials and Methods | 106 |
| 5.2.1 | Determination of inhibitory actinomycin D concentration | 106 |
| 5.2.2 | <i>MDR1</i> mRNA stability determination | 106 |
| 5.2.3 | <i>MDR1</i> RT-PCR | 107 |
| 5.3 | Results | 108 |

| | |
|--|------------|
| 5.3.1 Optimal actinomycin D concentration | 108 |
| 5.3.2 <i>MDR1</i> mRNA half life | 108 |
| CHAPTER 6 Discussion | 114 |
| 6.1 MDR and its reversible induction in K562 MDR sublines | 114 |
| 6.2 Transcriptional regulators of MDR1 | 117 |
| 6.3 GC box binding protein involvement in <i>MDR1</i> activation | 121 |
| 6.4 NRE binding protein involvement in <i>MDR1</i> activation | 122 |
| 6.5 <i>MDR1</i> transfection studies | 123 |
| 6.5.1 <i>MDR1</i> promoter studies | 123 |
| 6.5.2 Downstream region | 123 |
| 6.6 Effect of TSA on MDR levels | 124 |
| 6.7 Methylation status of the <i>MDR1</i> promoter | 125 |
| 6.8 Stability of <i>MDR1</i> mRNA in K562 MDR sublines | 126 |
| 6.9 Future directions | 128 |
| 6.10 Conclusions | 130 |
| References | 132 |

LIST OF FIGURES AND TABLES

| | | |
|-------------|--|----|
| Figure 1.1 | Structure of P-gp | 6 |
| Table 1.1 | Representative compounds that interact with P-gp | 8 |
| Figure 1.2 | The <i>MDR1</i> promoter structure | 17 |
| Figure 2.1 | Resistance levels of K/EC32 cells | 36 |
| Figure 2.2 | Resistance levels of K/EPR cells | 38 |
| Figure 2.3 | Resistance levels of K/DNR cells | 39 |
| Figure 2.4 | Northern blot for the determination of <i>MDR1</i> mRNA levels in K562 MDR cell lines | 41 |
| Table 2.1 | Normalised <i>MDR1</i> mRNA levels in K562 MDR cell lines | 41 |
| Figure 2.5 | Comparison of the <i>MDR1</i> gene copy number in K562 MDR cell lines | 42 |
| Table 2.2 | Normalised <i>MDR1</i> gene copy number in K562 MDR cell lines | 42 |
| Figure 2.6 | Stability of MDR | 43 |
| Figure 2.7 | The effect of 3 day drug exposure on the K/EC32 cell line | 45 |
| Figure 2.8 | The effect of short-term drug exposure on the K/EC32 and K/EC32T cell lines | 47 |
| Figure 2.9 | The effect of 3 day drug exposure on the K/EPR cell line | 48 |
| Figure 2.10 | The effect of 3 day drug exposure on the K/DNR cell line | 49 |
| Figure 2.11 | The effect of 3 day drug exposure on the K562 cell line | 50 |
| Figure 2.12 | Northern blot for the determination of <i>MDR1</i> mRNA levels in K562 MDR cell lines | 52 |
| Table 2.3 | Correlation of <i>MDR1</i> mRNA and relative resistance levels in K562 MDR sublines following 3 day drug treatment | 53 |
| Figure 2.13 | The effect of short-term epirubicin exposure on the K/EPR cell line | 55 |
| Figure 2.14 | The effect of short-term paclitaxel exposure on the K/EPR cell line | 56 |
| Table 2.4 | Comparison of K/EPR cell paclitaxel IC ₅₀ 's before and after 4 or 16 hour epirubicin or paclitaxel treatment | 57 |

| | | |
|--------------|--|-----|
| Figure 2.15 | The effect of short-term cisplatin exposure on the K/EPR cell line - paclitaxel cytotoxicity | 59 |
| Figure 2.16 | The effect of short-term cisplatin exposure on the K/EPR cell line – vinblastine cytotoxicity | 60 |
| Figure 2.17 | The effect of short-term rifampacin exposure on the K/EPR cell line | 62 |
| Figure 2.18 | Effect of TSA on growth and epirubicin resistance of K562 cells | 64 |
| Figure 2.19 | Effect of TSA on growth and epirubicin resistance of K/EPR cells | 65 |
| Figure 3.1 | EMSA for Y-box binding probe | 77 |
| Figure 3.2 | EMSA for (a) Y-box binding probe and (b) USF binding probe | 78 |
| Table 3.1 | Normalised Y-box binding factor levels in K562 MDR sublines | 82 |
| Figure 3.3i | EMSA using (a) single stranded Y-box binding oligonucleotide and (b) Supershift of Y-box binding factor with anti-NF-YA antibody | 82 |
| Figure 3.3ii | EMSA for supershifting of the Y-box factor with anti NF-YA | 83 |
| Figure 3.4 | EMSA for (a) USF and (b) –55GC box binding factors | 84 |
| Table 3.2 | Normalised –55GC box binding factor levels in K562, K/EPR and K/EPR drug treated cells | 86 |
| Figure 3.5 | Relative <i>MDR1</i> promoter activity in K562 and K/EPR cells | 87 |
| Figure 3.6 | <i>MDR1</i> promoter activity in K/EPR untreated and K/EPR treated cells | 89 |
| Figure 3.7 | EMSA for (a) NRE binding factor and (b) USF | 90 |
| Table 3.3 | Normalised NRE binding factor levels in K562, K/EPR and K/EPR drug treated cells | 90 |
| Figure 4.1 | Sequence of the <i>MDR1</i> promoter | 99 |
| Table 4.1 | Methylation status of CpG sites in the <i>MDR1</i> promoter region (- 462 to +782bp) in K562 and K/EPR cells | 100 |
| Figure 4.2 | EMSA for (a) binding to CpG sites 43 & 44 and (b) USF binding sequence | 102 |
| Figure 4.3 | Transcription factor analysis of sequence surrounding CpG sites 43 and 44 | 103 |

| | | |
|------------|--|-----|
| Table 4.2 | Transcription factor binding sequences | 103 |
| Figure 5.1 | Optimal actinomycin D concentration for K562 and K/EPR cells cell lines | 109 |
| Figure 5.2 | The effect of short-term drug exposure on the K562 and K/EPR | 110 |
| Figure 5.3 | <i>MDR1</i> mRNA half-life for K/EPR cells before and after Epirubicin treatment | 112 |
| Table 5.1 | Comparison of <i>MDR1</i> cDNA levels and paclitaxel resistance before and after 3 days epirubicin treatment. | 113 |

ABBREVIATIONS

| | |
|-------------------|---|
| A | adenine or adenosine |
| ABC | ATP-binding cassette |
| AMP | adenosine monophosphate |
| ATCC | American type culture collection |
| ATP | adenosine triphosphate |
| 5'-azadC | 5'-azadeoxycytidine |
| bp | base pairs |
| BSA | bovine serum albumin |
| C | cytosine or cytidine |
| CAT | chloramphenicol acetyl transferase |
| cAMP | cyclic AMP |
| cDNA | complementary DNA |
| Ci | curie |
| CO ₂ | Carbon dioxide |
| CpG | CG dinucleotide |
| CRE | cAMP regulator elements |
| CREB | cAMP-responsive element binding protein |
| CRS | cAMP response sequences |
| CTP | cytosine triphosphate |
| DEPC | diethylpyrocarbonate |
| DH ₂ O | deionised water |
| DMSO | dimethylsulphoxide |
| DNA | deoxribonucleic acid |
| DNase | deoxyribonuclease |
| dNTP | (unspecified) deoxyribonucleotide triphosphate |
| dsDNA | double stranded DNA |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetracetic acid |
| Egr | early growth response |
| EGTA | ethylene glycol-bis-(β -aminoethyl ether) N, N, N', N'-tetracetic acid |

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| EMSA | electrophoretic mobility shift assay |
| ET-743 | Ecteinascidin-743 |
| EtBr | ethidium bromide |
| 5-FU | 5-Fluorouracil |
| fmole | femtomole |
| g | grams |
| G | guanine or guanosine |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GSH | glutathione |
| GST | glutathione S-transferase |
| GTP | guanine triphosphate |
| HEPES | N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid |
| HSRE | heat shock response element |
| IC50 | 50% inhibitory concentration |
| kb | kilobase |
| kDa | kilodalton |
| L | litre |
| MDR | multidrug resistance |
| MDR1 | multidrug resistance gene 1 |
| MDR2 | multidrug resistance gene 2 |
| mM | millimolar |
| $\mu\text{J}/\text{cm}^2$ | microjoules per centimetre squared |
| μM | micromolar |
| ml | millilitre |
| mRNA | messenger RNA |
| MRP | multidrug resistance associated protein |
| MRP1 | multidrug resistance associated protein gene 1 |
| MTT | 3-4,5-dimethylthiazole-2,5 diphenyl tetra bromide |
| MW | molecular weight |
| nm | nanometer |
| nM | nanomolar |
| NRE | negative response element |

| | |
|---------|--|
| P/CAF | CREB binding protein associated factor |
| PCR | polymerase chain reaction |
| P-gp | p-glycoprotein |
| PKA | type 1 cAMP-dependant kinase |
| PKC | protein kinase C |
| pmole | picomole |
| PABP | poly(A)-binding protein |
| poly(A) | polyadenylated |
| RB | retinoblastoma |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RT | room temperature |
| RT-PCR | reverse-transcriptase polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SRE | stress response elements |
| SSC | sodium chloride sodium citrate |
| SSPE | Sodium chloride sodium phosphate + Ethylenediaminetetraacetic acid |
| SSRE | serum starvation response element |
| ssYB-1 | single stranded YB-1 |
| T | thymine or thymidine |
| TBE | Tris-borate buffer |
| TBP | TATA binding protein |
| TE | Tris EDTA |
| TPA | 12-O-tetradecanoylphorbol-13-acetate |
| Tris | tris(hydroxymethyl) aminomethane |
| TSA | trichostatin A |
| TSS | transcription start site |
| tRNA | transfer RNA |
| TTP | thymidine triphosphate |
| U | unit |
| UV | ultraviolet |

ABSTRACT

A major problem in chemotherapy is that many cancers are intrinsically drug resistant or later become resistant. Resistance to one drug is often accompanied by cross-resistance to many unrelated drugs and this is known as multidrug resistance (MDR). MDR is commonly associated with a 170kDa glycoprotein called P-glycoprotein (P-gp), which is thought to act as an ATP-dependant drug efflux pump and which is encoded for by the *MDR1* gene in humans. *MDR1* transcription can be initiated by various mechanisms such as demethylation of *MDR1* promoter sequences or translocation of the *MDR1* gene. In cells already expressing P-gp this expression can be increased by mechanisms such as amplification of the *MDR1* gene copy number or an increase in the rate of mRNA translation or stability. However the focus of many studies is on the regulation of *MDR1* transcription through the binding of transcription factors to specific sequences in the *MDR1* promoter region, in particular an inverted CCAAT element known as the Y-box and the -55GC box.

The current study investigated the mechanisms for MDR in a series of K562 derived MDR cell lines demonstrating varying levels of low level MDR. Levels of resistance of each of the K562 MDR cell lines were all confirmed by performing vinblastine and paclitaxel cytotoxicity assays on the cell lines. Northern hybridisation of total RNA isolated from K562 MDR cells indicated a positive correlation existed between the level of *MDR1* mRNA expressed in the MDR cells and the level of MDR displayed by the cells. Southern blot hybridisation of DNA from the cells with an *MDR1* probe indicated that increase of *MDR1* mRNA in the K562 MDR cells was not due to amplification of the *MDR1* gene. Bisulphite genomic sequencing of K562 and MDR cell lines revealed the *MDR1* promoter in both cell lines to be almost completely unmethylated apart from two distinct sites of methylation in K562 cells at two CpG sites downstream of the transcription start at +421 and +423bp respectively.

Treatment of the K562 cells with the histone deacetylase inhibitor Trichostatin A (TSA) increased the cells resistance to epirubicin, however no effect was seen upon TSA treatment of the K562 MDR cells. This suggests there is a difference in the chromatin structure in the two cell lines.

It was further demonstrated that resistance levels of K562 derived MDR cell lines declined with increasing time in drug free culture, but could be restimulated by short term drug exposure to P-gp substrate drugs, in some cases in as little as after 4 hours exposure, suggesting a transcriptional mechanism of *MDR1* upregulation is likely to be responsible for the induction. Consistent with this, mRNA stability studies indicated that the drug induction of K562 MDR cells that resulted in increased MDR and *MDR1* mRNA levels was not mediated by an increase in the stability of *MDR1* mRNA, as the rate of mRNA decay was the same in treated and untreated control cells. However, electrophoretic mobility shift assays (EMSA) of the two major transcription factors involved in *MDR1* regulation, the Y-box binding protein (identified as NF-Y) and the – 55GC box binding protein indicated no difference in nuclear levels of these two proteins in untreated and drug induced cells. Thus the mechanism for up-regulation of *MDR1* activity in K562 MDR cell lines is most likely due to activation via alteration of other factors or via changes in chromatin structure regulating accessibility of transcription factors.

CHAPTER 1

Introduction

1.1 Cancer

Cancer is a leading cause of death in industrialised society, resulting in over six million deaths per year (Ferley *et al.*, 2001). The process of carcinogenesis is a multistage process, with the emergence of a cancerous cell population the result of the sequential accumulation of alterations in genes responsible for the control of cellular proliferation, cell death and the maintenance of genetic integrity (Vainio *et al.*, 1992; Balmain and Harris, 2000).

Cancer is generally treated by surgery, radiotherapy, chemotherapy, or a combination of the three (Hellman and Vokes, 1996). Surgery is the oldest and most widely used treatment and with advances in new technology minimising invasive surgery it can be quick and effective (Bremers *et al.*, 1999). However surgery is unable to treat cancers that have metastasised throughout the body and is also not appropriate to treatment for tumours that are attached to vital organs or for malignant disorders of the blood. Radiotherapy involves exposing dividing cancer cells to ionising radiation and aims at inflicting cellular damage sufficient to cause cell death (Price and Sikora, 2000). Although not always fully effective, radiotherapy is often safer for elderly patients who may be unable to recover from the trauma of surgery, and like surgery, is inappropriate for the treatment of metastatic tumours. Chemotherapy, another treatment strategy, involves the systemic administration of an anticancer drug with the aim of achieving selective toxicity to cancer cells by targeting differences between normal and cancer cells. Chemotherapy has the advantage of being effective against widely disseminated

tumour types (Hellman and Vokes, 1996). This treatment has been in use since the 1940s and usually involves interference with tumour cell replication and/or transcription (Hellman and Vokes, 1996; Gottesman, 2002). During this time over one hundred cytotoxic agents have been approved for the treatment of cancer however the efficacy of chemotherapy has been hampered by the occurrence of dose limiting side effects and the development of drug resistance.

1.2 The Problem of Multidrug Resistance in Cancer

The aim of chemotherapy is to eliminate cancer cells. This has been successfully achieved in some cases by the use of various combinations of anti-neoplastic drugs (Ling, 1994). However a major problem of chemotherapy is that many forms of cancer are intrinsically refractory to drug treatment and others that initially respond, later become resistant (Ling 1994). Although there are many factors that may lead to the failure of chemotherapeutic treatment of cancer, it is thought that one of the main contributors is the development of drug resistance at the cellular level (Rischin and Ling, 1993). It has also been found that resistance to one drug is often accompanied by cross-resistance to a broad range of structurally and functionally unrelated agents, a phenomenon known as multidrug resistance (MDR) (Rischin and Ling, 1993). MDR was originally characterised by reduced drug cytotoxicity due to the decreased intracellular accumulation of several drugs (for review see Kane, 1996). MDR cells are typically resistant to small hydrophobic, natural product drugs such as anthracyclines (doxorubicin, daunorubicin, epirubicin and idorubicin), *Vinca* alkaloids (vincristine and vinblastine), epipodophylotoxins (etoposide and teniposide), taxanes (paclitaxel and taxotere) and amsacrine (Sonneveld, 2000; Kane, 1996). In some cancers an extended form of MDR may also be seen where cells are resistant to additional non-classical MDR drugs such as methotrexate and chlorambucil (Marks *et al.*, 1996).

Many different drug resistant mechanisms have been attributed to the development of MDR and rather than operating singularly, it is most likely that many of these mechanisms operate concurrently. Much of MDR research has focused on the overexpression of drug transporter proteins, for example P-glycoprotein (P-gp) (for

review see Harrison, 1995; McKenna, 1997; Ross, 2000) and the multidrug resistance-associated protein (MRP) (for reviews see Borst, 1999; Ishikawa, 2000; Leslie, 2001). P-gp and MRP are both members of the ATP-binding cassette (ABC) superfamily of transporters and are involved in the extrusion of cytotoxic drugs from cells and thereby reducing intracellular drug accumulation and hence decreasing the cytotoxic effect on the cells. P-gp and MRP exhibit similar multidrug resistance phenotypes, for example they both confer resistance to *vinca* alkaloids, anthracyclines and epipodophylotoxins, however P-gp is able to bestow greater resistance to taxanes (Kane, 1996; Leslie, 2001). The taxane paclitaxel is a good indicator of P-gp mediated MDR as it is not a substrate for MRP (Breuninger *et al.*, 1995; Grant *et al.*, 1994). Paclitaxel specificity for P-gp over MRP has been demonstrated in NIH/3T3 MRP transfectants (Breuninger *et al.*, 1995). In this study, MRP transfected cells displayed increased resistance to several lipophilic drugs, however increased resistance was not observed for paclitaxel (Breuninger *et al.*, 1995).

MRP1, one of the 6 functionally characterised genes for MRP, effluxes drugs *in vivo* via a co-transporter mechanism with reduced glutathione (GSH) (Harrison, 1995; Ishikawa, 2000). Therefore it is probable that the amount of available GSH and associated GSH-dependant enzymes will affect drug resistance. The overexpression of these conjugation enzymes, particularly glutathione S-transferase (GST), has been seen in many human cancers (Gekeler, 1992; Gurbuxani, 2001; Harrison, 1995). GST overexpression has also been associated with increased P-gp expression and it is thought that GST may present MRP with its substrate (Harrison, 1995).

Another possible mechanism causing MDR is qualitative or quantitative alterations to the enzymes targeted by cytotoxic drugs. Topoisomerases are a group of enzymes involved in transcription, DNA repair and cell proliferation by breaking and re-ligating DNA strands. Alterations in expression or function of these enzymes, for example topoisomerase II, have been associated with MDR (for reviews see Harrison, 1995; Lohri, 1997; McKenna, 1997).

Many chemotherapeutic drugs function by inducing apoptosis (programmed cell death), for example etoposide, VM26, m-AMSA, dexamethasone, vincristine, cisplatin, cyclophosphamide, paclitaxel, 5'-fluorouracil (5-FU) and adriamycin (Hannun, 1997).

However many cancers show intrinsic resistance to apoptosis, or acquire resistance upon chemotherapeutic drug treatment, thus rendering them MDR. This is thought to arise due to mutation or defects in cells' apoptotic signal pathways or regulatory mechanisms such as the p53 tumour suppressor protein. Functional p53 acts as a transcription factor that regulated the expression of a large number genes involved in cell cycle control, the induction of apoptosis, genetic stability, DNA repair, differentiation control and angiogenesis (reviewed in Stewart and Kleihues, 2003; Vogelstein *et al.*, 2000; Carr *et al.*, 2000; El-Deiry *et al.*, 1998). The p53 tumour suppressor gene is the most frequent gene mutated in cancer (Greenblatt *et al.*, 1994; Hollstein *et al.*, 1991) and is thought to be mutated in over 50% of human cancers (Hartwell *et al.*, 1994; Shaw *et al.*, 1992; Lane and Fischer, 1996). Its association with tumour development is evident in p53 knockout mice, which show a high incidence of tumour development at an early age (Donehower, 1996). In addition, germ line mutation of one p53 allele in humans gives rise to the Li-Fraumeni cancer susceptibility syndrome (Donehower, 1996; Srivastava *et al.*, 1990). The induction of apoptosis in cancer cells is a common mechanism of action of a number of chemotherapeutic drugs, consequently loss of the p53 apoptotic pathway may influence the sensitivity of cells to the drug. *In vitro* studies have reported that loss of p53 function reduced cellular sensitivity to 5-FU, if not abolishing it entirely (Bunz *et al.*, 1999; Longley *et al.*, 2002; Hwang *et al.*, 2001). For example one study by Bunz *et al.*, (1999) found that loss of functional p53 in colon carcinoma cells made the cells resistant to apoptosis by 5-FU compared to parental cells with wild type 53. In addition, p53 null mice were found to be resistant to apoptosis triggered by 5-FU (Pritchard *et al.*, 1998).

Mutations or gene translocations of anti-apoptotic genes such as *bcl-2* and *bcl-X1*, can lead to protein overexpression and thus resistance to apoptosis (Chresta, 1999; Clynes, 1998; Hannun, 1997; Pan, 1997). An example of this is seen in chronic myeloid leukaemia whereby greater than 90% of patients demonstrate the Philadelphia chromosome, that is, a reciprocal chromosomal translocation of t(9:22) resulting in the generation of a *bcr/abl* translocated gene (Wu *et al.*, 1999; Langabeer *et al.*, 2002; Sonoyama *et al.*, 2002). The *abl* gene encodes c-abl tyrosine kinase which induces apoptosis in response to DNA damage. However, the fusion protein that results from the *bcr/abl* translocation acts as an oncogene and confers apoptotic resistance to DNA damage (for reviews see Skorski, 2002a; Skorski, 2002b). Studies demonstrated the

bcr/abl translocation enables the proliferation of haematopoietic cells under deprived conditions (Sonoyama *et al.*, 2002) and prolongs cell cycle arrest at the G2M phase (Nishii *et al.*, 1996).

1.3 P-glycoprotein mediated MDR

Studies using *in vitro* drug resistant cell lines have indicated that a vast array of mechanisms can contribute to overall MDR. However, research has shown that a major contributor to drug resistance is the expression of the 170kDa transmembrane glycoprotein drug transporter P-glycoprotein (P-gp) (for review see Harrison, 1995; McKenna, 1997; Ross, 2000). P-gp belongs to the ATP-binding cassette (ABC) superfamily of transporters and is thought to act as an ATP-dependant membrane efflux pump (for review see Baggetto, 1997).

P-gp has been predicted to be composed of two homologous halves, each containing six putative α helix trans-membrane domains and an ATP binding domain (Figure 1.1a and b). These two halves of P-gp are thought to be joined by a linker region and evidence has shown that both nucleotide binding sites, which are contained on the C-terminus of each half and responsible for ATP binding and hydrolysis, are necessary for transport of P-gp substrates out of the cell (Ambudkar *et al.*, 1999). The dependence P-gp activity on ATP was determined by mutational analysis on these C-terminus ATP binding domains, whereby mutations resulted in cessation of P-gp activity (Ruetz *et al.*, 1994). Some controversy over the secondary structure of P-gp exists and a more recent “dual channel model” has been proposed by Jones and George (2000) and is shown in figure 1.1c. This model proposes that the substrate binding site is formed from large intracellular loops which attach α -helix bundles to the transmembrane domain which consists of two β barrels. ATP binding sites are thought to be beneath and between these β barrels (Jones and George, 2000)

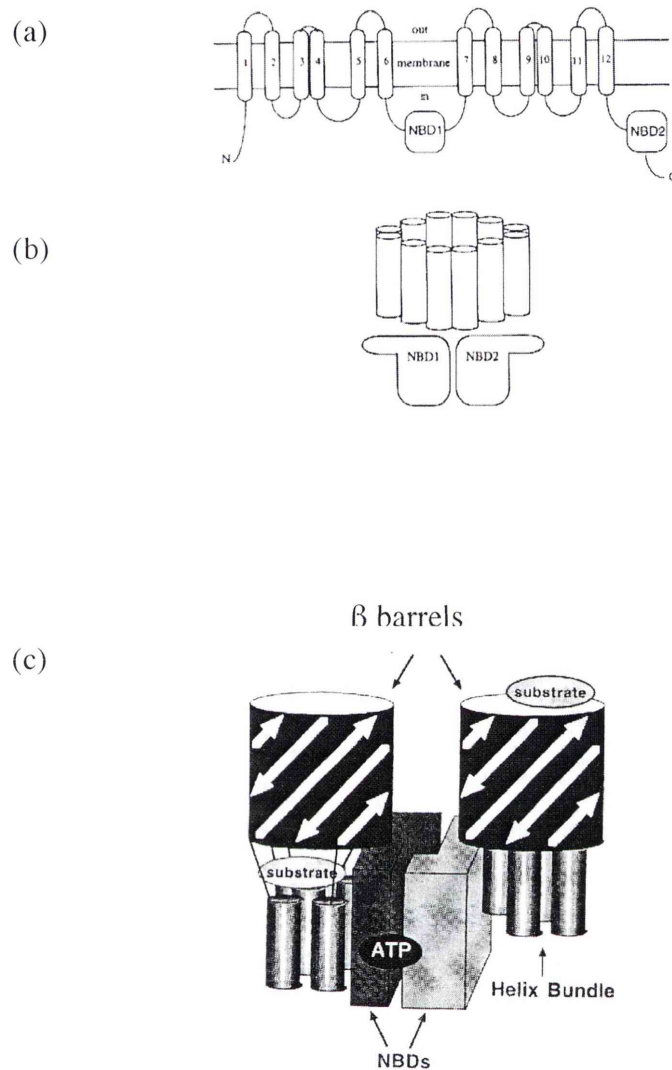


Figure 1.1 Structure of P-gp (a) Linear representation of the human P-glycoprotein inserted within the cell membrane showing the twelve putative α helix transmembrane regions, ATP binding domains (NBD1 and NBD2), (b) Tertiary structure model showing ring of helices forming a single membrane channel (taken from Jones and George, 2000), (c) Alternative model proposed by Jones and George (2000) with double β barrel transmembrane domain.

Two classes of P-gp, encoded by the genes *MDR1* and *MDR2*, have been identified in the human genome (Chin *et al.*, 1989). However experiments where *MDR1* or *MDR2* cDNA have been transfected into drug sensitive cells have demonstrated that only the *MDR1* encoded P-gp bestows an MDR phenotype (Ueda *et al.*, 1989, Roninson *et al.*, 1986).

Cell lines that express P-gp are able to take up cytotoxic drugs but their cellular accumulation is limited by the rate of their drug efflux by P-gp and hence by the level of expression of P-gp (Chen & Simon, 2000). P-gp is able to efflux *vinca* alkaloids, anthracyclines and epipodophylotoxins, taxoids as well as other anti-neoplastic drugs such as actinomycin D. Examples of some of the many different classes of compounds that interact with P-gp are listed in Table 1.1.

P-gp mediated drug resistance has been shown to be reversed by agents known as resistance modulators or chemosensitising reagents. There are several classes of modulators of P-gp, these include calcium channel blockers, immunosuppressive agents, *vinca* alkaloid analogues, steroidal agents, cyclosporin analogues, calmodulin antagonist and other miscellaneous hydrophobic agents (Clynes, 1998). P-gp modulation mechanisms are not fully understood and may differ for different compounds. However, the three main mechanisms by which modulators are thought to act are (i) via the blockage of drug transport by direct high affinity binding with the P-gp binding sites through competitive or non-competitive interaction, and therefore inhibiting the binding of cytotoxic drugs; (ii) via inhibition of ATP binding, ATP hydrolysis or coupling of ATP hydrolysis to the translocation of the substrate; (iii) via disruption of the cells lipid membrane through interaction with the membrane environment or modification of drug-membrane interaction (Ambudkar, *et al.*, 1999; Muller *et al.*, 1995). The main concern with P-gp mediators is that many are toxic at required doses and are therefore not suitable for therapeutic use *in vivo*. Even some of the more commonly clinically used modulators such as verapamil, can cause detrimental side effects such as cardiac problems (Ozols, 1987).

The reversal of drug resistance by verapamil in *in vitro* cytotoxicity assays is useful as it is a good indicator that P-gp mediated drug resistance is operational in a cell line.

Table 1.1 Representative compounds that interact with P-glycoprotein (taken from Ambudkar *et al.*, 1999)

Anticancer drugs

Vinca alkaloids (vincristine, vinblastine)

Anthracyclines (doxorubicin, daunorubicin)

Epipodophyllotoxins (etioposide, teniposide)

Taxol (paclitaxel, docetaxel)

Actinomycin D

Mitomycin C

Topotecan

Mithramycin

Other cytotoxic agents

Colchicine

Emitine

Puromycin

Calcium channel blocker

Diltiazem

Felodipine

Verapamil

Nicarpedine

HIV protease inhibitors

Ritinovir

Indinavir

Saquinavir

Antifungals

Ketaconazole

Itraconazole

Hormones

Hydrocortisone

Progesterone

Testosterone

Dexamethasone

Estradiol

Immunosuppressants

Cyclosporine

FK506

Rapamycin

Detergents

TritonX-100

Tween 80

Solutol HS-15

Hydrophobic peptides

Gramicidin D

Valinomycin

N-acetyl-leucyl-leucylnorleucine

Other compounds

Digoxin

Tamoxifen

Terfenadine

Rhodamine 123

Calcein-AM

Morphine

Although the exact mechanism of how verapamil reverses P-gp mediated MDR is not fully understood, it is thought that verapamil itself is a substrate for P-gp and binds with high affinity directly to P-gp. This binding of verapamil to P-gp inhibits the binding of cytotoxic drugs thus, the drugs are not excluded from the cell (Clynes *et al.*, 1998; Muller *et al.*, 1995; Gottesman and Pastan, 1989; Cano-Gauci, and Riordan, 1987). Verapamil is significantly less capable of reversing drug resistance in cell lines that overexpress MRP as, unlike P-gp, verapamil does not directly interact with MRP (Clynes *et al.*, 1998; Breuninger *et al.*, 1995; Coley *et al.*, 1991; McGrath *et al.*, 1989). McGrath *et al.* (1989) demonstrated this by using an ¹²⁵I-labeled photoaffinity analog of verapamil to label proteins associated with drug resistance in the cell membranes of the cell lines HL60/Vinc, which overexpress P-gp, and HL60/Adr, which overexpress MRP. In this study it was shown that only the P-gp containing membranes of HL60/Vinc cells were labelled and no selective labelling occurred in the membranes of the HL60/Adr cells.

1.3.1 P-glycoprotein expression in normal tissues

Studies have demonstrated *MDR1* gene expression in many normal tissues. A high level has been found in the apical membrane of the intestinal epithelial cells of both the small and large intestines, adrenal gland, biliary canalicular membrane of hepatocytes and in the luminal membrane of proximal tubular epithelial cells in the kidney (Croop, 1989; Goldsmith, 1993; Madden, 1993; Morrow, 1994; Thiebaut, 1987). It is also abundant in the luminal membrane of the endothelial cells lining the small blood capillaries in the brain and testes (Cordon-Cardo, 1989; Madden, 1993). Moderate levels are also seen in the luminal membrane of the endometrium of pregnant uterus (Arceci, 1988; Madden, 1993) and in CD34-positive bone marrow cells, normal peripheral blood lymphocytes and hematopoietic stem cells (Chaudhary, 1991).

Although the precise function of the *MDR1* gene product, P-gp, is unknown, the pattern of gene expression is indicative of a physiological role in the transport of organic molecules, such as toxins or steroids, from normal cells and tissues. In an effort to

confirm a physiological importance for P-gp, Schinkel *et al.* (1998) developed P-gp knockout mice. The two genes, *MDR1a* and *MDR1b*, that encode P-gp in mice together show a combined tissue distribution similar to that of *MDR1* in humans. The P-gp knockout mice showed no obvious physiological or anatomical abnormalities and also appeared biochemically and haematologically normal. However when these mice were challenged with drugs, they demonstrated increased drug accumulation in the brain and central nervous system, and decreased intestinal and biliary excretion. Therefore it was concluded that although P-gp appeared not to be essential for basic physiological functions, it does play an important role in forming a barrier in the protection of sensitive organs, such as the brain and the intestines from toxin penetration (Schinkel, 1998; Schinkel, 1999).

1.3.2 P-glycoprotein expression in cancer *in vivo* and *in vitro*

P-gp has been demonstrated in many human cancers. In some cases these cancers are derived from tissues in which *MDR1* is normally expressed, such as those mentioned above. In other cases where the cancer is derived from tissues not normally expressing P-gp, the cancer expresses P-gp either after exposure to chemotherapeutic drugs or during carcinogenesis (Gottesman and Pastan, 1993).

There is a vast quantity of literature which correlates *MDR1* expression and P-gp activity with MDR in culture model systems. A correlation between P-gp expression and clinical MDR has been observed, particularly for haematological malignancies (Pirker *et al.*, 1991, Leith, 1995; te Boekhorst, 1995), and to a lesser extent solid tumours (Schneider *et al.*, 1989; Cheng *et al.*, 1993; Coley *et al.*, 2000; Ng *et al.*, 2000). There is also some suggestion of a correlation between patient prognosis and P-gp expression. This has been demonstrated in both haematological malignancies, such as acute myeloid leukaemia (Borg *et al.*, 1998; Senent *et al.*, 1998), promyelocytic leukaemia (Michieli *et al.*, 2000), acute non-lymphocytic leukaemia (Damiani *et al.*, 1998), and myelodysplastic syndromes (Wattel *et al.*, 1998) and in solid malignancies such as hepatocellular carcinomas (Ng *et al.*, 2000) and soft tissue sarcomas (Coley *et al.*, 2000).

Development of MDR cancers may occur in several progressive steps, from initial activation of expression in the case of cancers arising from non-Pgp expressing tissues, to stepwise increases in *MDR1* activation following relapse from chemotherapy and progression to a more aggressive drug resistant cancer. These may involve multiple different activation mechanisms resulting in stable changes in phenotype. In cancer cells where P-gp does not appear to be expressed prior to drug treatment or is expressed at low levels, some event must occur that initiates or up-regulates its expression. Possible such events that may initiate *MDR1* transcription are changes in the methylation status of the gene's promoter or other regulatory sequences (Desiderato, 1997; Kantharidis *et al.*, 1997) or by gene rearrangement, where *MDR1* is translocated downstream of an active promoter (Mickley *et al.*, 1997). When the *MDR1* gene is already expressed, increase of P-gp expression can result from amplification of the *MDR1* gene (Morrow *et al.*, 1994). An increase in the rate of translation or stability of *MDR1* mRNA has also been reported to cause upregulation of P-gp expression (Marino *et al.*, 1990; Yague *et al.*, 2003).

An additional phenotype that has been observed in studies of cultured cells is reversible induction of *MDR1* activation. This occurs for cultured cell lines that require continuous or intermittent repeated low dose exposure to the selecting drug to maintain MDR. Such inducible MDR mechanisms need to be distinguished from stable changes in *MDR1* activation states. For example the CEM/A7R MDR lymphoid leukaemic cell line exhibits decreased P-gp expression and decreased MDR when grown for an extended period of time in the absence of drug, however levels of P-gp expression can be induced and drug resistance levels restored by short term drug exposure (Hu *et al.*, 1995; Marino *et al.*, 1990; Yague *et al.*, 2003).

A complication of studying the role of P-gp in MDR *in vivo* is the detection of P-gp. Several techniques have been employed for this purpose, including RNA and antibody based methods. However, while each appears to have its advantages and disadvantages there are conflicting views over which technique is the best or most sensitive. A further complication is that the definition of the level of P-gp or *MDR1* expression that is significant for the development of MDR varies among different research groups, even when using the same techniques (Ferry, 1998).

Monoclonal antibodies against different epitopes of P-gp have been used for P-gp detection by immunohistochemistry or flow cytometry. While some believe the use of monoclonal antibodies to detect P-gp is a principally reliable method (Pearson and Cunningham, 1993), there is also evidence to suggest otherwise. One monoclonal antibody commonly used in P-gp detection is MRK-16. This antibody recognises an extracellular epitope of P-gp and it is this feature that gives it its advantage over other P-gp specific monoclonal antibodies. Firstly, MRK-16 can recognise P-gp on live cells with no fixation required and therefore can be incorporated easily into flow cytometric analysis (Rischin and Ling, 1993). Secondly, as the epitope detected is external it is thought that only functional P-gp on the surface is measured (Hamada and Tsuruo, 1986). However, in a study whereby clinical samples from patients with chronic lymphocytic leukaemia were tested for P-gp expression using MRK-16 monoclonal antibody and by measuring the *MDR1* mRNA expression, 12% of the samples were indicated to be P-gp positive using MRK-16, whereas 53% showed increased *MDR1* mRNA (Cumber *et al.*, 1990). The experiment was then repeated but this time the samples were treated with neuraminidase prior to treatment with MRK-16. The results from this showed 52% of samples with P-gp expression using the MRK-16 antibody, which is approximately equal to the percentage of samples with increased *MDR1* mRNA. From this it would appear that the addition of neuraminidase before MRK-16 unveils epitopes that were previously masked.

Contradicting the study by Cumber *et al.* (1990), are the results from a series of experiments that were done by Ludescher *et al.* (1992). Here, Rhodamine 123, a fluorescent dye transported by P-gp, was used in flow cytometry to analyse MDR in samples from six acute myeloid leukaemia patients. Rhodamine 123 efflux is considered to indicate the presence of P-gp. Three of the six samples exhibited a marked decrease in accumulation of Rhodamine 123. Reverse-transcriptase PCR (RT-PCR) measurement of *MDR1* mRNA and MRK-16 staining was carried out on the three samples showing Rhodamine 123 efflux. Only one of the three samples showed positive for P-gp by detection with MRK-16, even though the method used to label the cells with MRK-16 was that used by Cumber *et al.* (1990), that is, the cells were treated with neuraminidase prior to MRK-16 incubation. All three samples were shown to have low to moderate levels of *MDR1* mRNA expression, thereby indicating that MRK-16 may not have the necessary sensitivity to detect lower levels of P-gp, and hence low level

MDR. Thus a poor correlation between P-gp expression and MDR *in vivo* may result when MRK-16 antibody is used to detect P-gp. RT-PCR appears to be a more suitable method for the detection of low level *MDR1* expression. However according to Pearson and Cunningham (1993), even this may not be accurate for determining the degree of P-gp expression, as the levels of protein in a cell do not always correspond with the expression of the mRNA transcript.

1.4 Mechanisms for the activation of P-gp

1.4.1 Methylation

It has been well documented that methylation of DNA plays an important role in the control of many genes such that methylation of specific DNA sequences can result in the silencing of a gene and demethylation can be associated with the activation of a previously transcriptionally silent gene (Bird, 1992). DNA methylation occurs on a cytosine in specific promoter CpG dinucleotides. In many genes CpG rich regions are found proximal to the promoter. These regions, known as CG islands, are not normally methylated with the exception of genomically imprinted and X-linked genes (Laird and Jaenisch, 1994). However, in the development of cancer, alterations to DNA methylation can occur (Schroeder and Mass, 1997). Studies have demonstrated a correlation between cancer development and tumour suppressor gene inactivation due to hypermethylation of the CG islands of these genes. Examples where tumour suppressor gene hypermethylation has been seen include the *RB* gene in retinoblastoma (Greger *et al.*, 1989), the *VHL* gene in renal cell carcinoma (Herman *et al.*, 1994) and the *Mts1* (Merlo *et al.*, 1995), p16 (Martinez-delgado *et al.*, 1997, Baur *et al.*, 1999) p53 genes (Schroeder and Mass, 1997) in many human cancers.

Evidence for a relationship between the demethylation of *MDR1* promoter sequences and initiation of *MDR1* expression in MDR cancer cells has also been reported. Kantharidis *et al.*, (1997) demonstrated differential methylation between drug sensitive and drug resistant cells derived from the CCRF-CEM human T-cells leukaemia cell line and in clinical samples from chronic lymphoblastic leukaemia patients. Southern

analysis was performed on *MDR1* restriction enzyme digestions using HpaII, a methylation sensitive enzyme, and MspI, a methylation insensitive enzyme, that both recognise the same CCGG consensus sequence. Results showed that some of the HpaII recognition sites were protected by methylation in the drug sensitive cells but were not protected in the drug resistant cells. In addition a second experiment was carried out where drug sensitive cells were treated and grown in 5'-azadeoxycytidine (5'-azadC), a drug that prevents DNA methylation. When Southern analysis of digestions were repeated, the HpaII sites that were originally protected became unmethylated. RT-PCR of these 5'-azadC treated cells also detected *MDR1* mRNA which was absent prior to 5'-azadC treatment. Thus, demethylation of the *MDR1* promoter correlates with activation of the *MDR1* promoter. Similarly, Desiderato *et al.*, (1997), using a technique which allows detection of the methylation status of all CpGs present, has shown extensive hypermethylation of sites both upstream and downstream of the *MDR1* transcription start site in the drug sensitive non-P-gp expressing HL60 cell line. In contrast, P-gp expressing MDR HL60 sublines were completely unmethylated in these regions.

Promoter methylation may serve to directly inhibit transcription through the methylation of specific CpG sites within transcription factor binding sites thus preventing the transcription factor binding and gene transcription. However it may also act indirectly to inhibit gene transcription by causing conformational changes in chromatin. In eukaryotic cells nuclear DNA is packaged into nucleosomes by histones and this is further condensed into chromatin (Kornberg, 1974). As well as enabling packaging of DNA, this hierarchal order also enables gene regulation through alterations in the level of DNA condensation. In genes transcribed at a specific point in the cell cycle or in a certain tissue type, chromatin is maintained in an active de-condensed state, whereas when genes are not expressed, chromatin is coiled in a highly inactive condensed state. (Baylin 1997).

Transcriptional repression can result if specific transcription factors are prevented from the binding to their consensus sequences when sequences are hidden due to steric hindrance from DNA being arranged in a closed chromatin structure (Levine *et al.*, 1991; Boyes and Bird, 1992; Jones *et al.*, 1998). DNA that is hypermethylated is associated with dense or closed chromatin organization and hence transcription inhibition. DNA that is hypomethylated is associated with open chromatin and active

transcription (Jones and Wolfe, 1999). Alteration of chromatin structure can occur through the action of methyl-CpG binding proteins. Two such proteins are MeCP1 (Meehan *et al.*, 1989; Antequera *et al.*, 1989) and MeCP2 (Pawlak *et al.*, 1991; Jost *et al.*, 1991) which bind to clusters of methylated CpG dinucleotides and single methylated CpG dinucleotides respectively. MeCP2 is thought to bind through its DNA binding domain to methylated CpG dinucleotides and silence transcription from a distance through its repressor domain (Nan *et al.*, 1993; Nan *et al.*, 1997). The repressor domain of MeCP2 has been shown to recruit complexes containing the Sin3A corepressor and histone deacetylase corepressor, these complexes direct the modification of chromatin into a more stable and functionally inert state (Jones *et al.*, 1998; Nan *et al.*, 1998).

Histones within the nucleosome are constantly undergoing acetylation and deacetylation. Protruding lysine residues in the positively charged NH₂-terminal tails are the primary target sites of acetylation. This is thought to lead to neutralisation of the positive charge resulting in allosteric changes in chromatin conformation and destabilisation and loosening of histone-DNA contacts which in turn increases transcription factor access to DNA (Yoshidai *et al.*, 2001; Wolffe, 1996). When histones become deacetylated the affinity of the histone tail for DNA increases and thus histone deacetylation is correlated to a closed chromatin structure and hence transcriptional silencing (Wolffe, 1996). The level of acetylation of histones within the nucleosome is a posttranslational modification catalysed by histone acetyltransferases and deacetylases (for reviews see Davie, 1997; Wade *et al.*, 1997).

1.4.2 Gene rearrangement

A further mechanism that may be involved in the activation of *MDR1* transcription resulting in P-gp expression is gene rearrangement. A study by Mickley *et al.*, (1997) demonstrates *MDR1* activation in adriamycin-selected S48-3s cells as a result of juxtaposition of the *MDR1* gene from chromosome 7 to an active promoter of a gene on chromosome 4. Similarly gene rearrangements were observed in two lymphoblastic leukaemia samples from patients refractory to drug treatment.

1.4.3 Gene amplification

Amplification of the *MDR1* gene copy number can be a factor in high *MDR1* expression (Morrow, 1994). Gene amplification frequently occurs *in vitro* during the selection of multidrug resistant cell lines. According to Kohno *et al.*, (1994), in early stages of stepwise selection of *in vitro* cell cultures with cytotoxic drugs *MDR1* expression is increased without gene amplification. However, in late stages gene amplification is observed. Gene amplification is especially seen when the cells are exposed to high concentrations of drugs and therefore may be avoided using lower, more clinically relevant drug levels to treat cells (Shen *et al.*, 1986). *MDR1* copy number and *MDR1* mRNA levels can be determined by Southern and Northern analysis respectively and comparisons of these results will demonstrate if increase in *MDR1* expression is due to gene amplification or an actual increase in *MDR1* mRNA (Kohno *et al.*, 1994).

1.4.4 Transcriptional activation

The human *MDR1* gene reportedly contains two different promoter regions, the downstream or proximal promoter and the upstream or distal promoter (Ueda *et al.*, 1987). In normal tissues and most tumours, the major site of *MDR1* transcriptional regulation has been found to be under the control of the proximal promoter located at -434 to +1 of the *MDR1* gene sequence (Cornwell and Smith, 1993a). A minor site of *MDR1* transcription is located within the distal promoter and is only used in a few cell lines for a percentage of their *MDR1* transcripts. The importance of this promoter is not well understood (Baggetto, 1997). For the purpose of this review the proximal is referred to as the *MDR1* promoter.

The structure of the human *MDR1* promoter, shown in Figure 1.2, does not contain a TATA box but has been found to contain many consensus binding sequences for transcription factor binding sites that might act as response elements and thus have a possible role in its regulation. However, despite extensive research the significance of many of these regulatory factors to the problem of MDR *in vivo* has yet to be

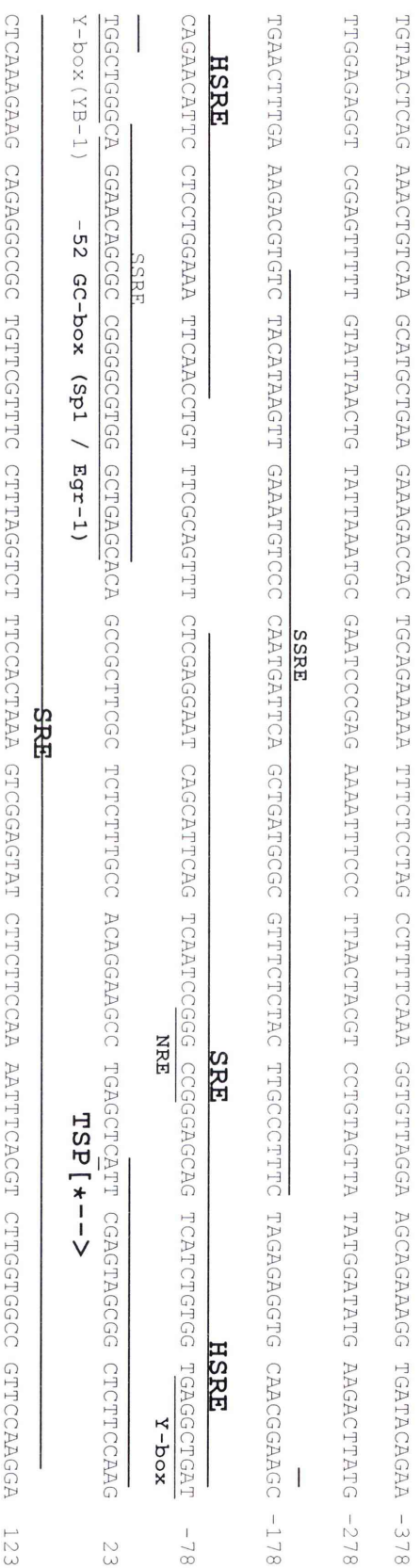


Figure 1.2 The *MDR1* promoter structure showing the position of various regulatory elements (underlined) as well as the Sp1/EGR-1 binding sequence at the -52 GC-box and the YB-1 binding sequence at the Y-box.

SSRE = serum starvation response element; HSRE = heat shock response element; SRE = stress response element; NRE= negative response element; TSP *= transcription start point.

determined. Moreover, the vast majority of studies on the *MDR1* promoter structure concentrate on the sequence upstream of the transcription initiation start point and very little work has been done downstream of this region. Several groups have used reporter gene systems, most commonly a chloramphenicol acetyltransferase (CAT) gene fused to and directed by the human *MDR1* promoter, to identify important transcriptional regulatory elements in the *MDR1* promoter. One such study by Miyazaki *et al.*, (1992) used human cancer KB cells transfected with the CAT reporter gene fused to the *MDR1* promoter to examine the effect of heat shock on the *MDR1* promoter activity. By incubating the cells at 45°C for 90 minutes it was found that CAT activity driven by the *MDR1* promoter became induced. Varying the length of this *MDR1* promoter in the reporter gene system suggested that the human *MDR1* promoter contained heat shock response elements (HSRE) at -178 to -152 and at -136 to -76 up from the transcription initiation start site (Miyazaki *et al.*, 1992).

A similar study by Tanimura *et al.*, (1992) also using KB cells transfected with CAT-*MDR1* promoter reporter gene system, showed an increase in *MDR1* promoter activity in response to serum starvation. A series of deletions in the *MDR1* promoter fused to the CAT gene identified a serum starvation response element (SSRE) in the sequence between -258 and -198 upstream of the initiation site. Cornwell and Smith, (1993b) identified a further SSRE in the sequence between -69 to -41 of the *MDR1* promoter. Interestingly, this region (-69 to -41) which is known as the -55 GC box, contains overlapping consensus binding sites for the transcription factors Sp1 and Egr1 (Cornwell and Smith, 1993a). This is one of several GC boxes found within the *MDR1* promoter, however the -55 GC box appears to be the most essential in terms of basal transcription (Cornwell and Smith, 1999a, Sundseth *et al.*, 1997). Evidence for this comes from mutation and deletion experiments on the region containing the -55 GC box. In the case of both deletion and mutation, a significant reduction in promoter activity was observed, and in one instance a point mutation in the -55 GC box gave a 60-70% decrease in promoter activity (Sundseth *et al.*, 1997).

Further indication as to the importance of this area was provided by *in vitro* DNase footprinting analysis which showed that this GC-box is a protected region (Cornwell and Smith, 1999a). Sp1 is an activator of many promoters and is believed to behave as an activator of *MDR1* transcription through binding at the -55 GC box. This has been

demonstrated by co-transfecting an *MDR1*-luciferase reporter gene with either an Sp1 cDNA construct or vector control into cells that do not express Sp1. Results from this experiment showed that the *MDR1* promoter was activated 15 fold higher in the presence of Sp1 compared to the vector only control construct (Cornwell and Smith, 1999a). High levels of Sp1 expression have been demonstrated in some human cancers, for example Kitadai *et al.*, (1992) demonstrated increased Sp1 mRNA expression in 12 out of 18 tumors at levels higher than normal mucosa. Likewise, Lietard *et al.*, (1997) found increased Sp1 levels in human hepatocellular carcinomas.

Egr-1 is a member of a family of transcription factors known as early growth response (Egr) factors. Recombinant Egr-1 has been shown to bind the -55 GC box region (Cornwell and Smith, 1999a) and participates in *MDR1* regulation in some phorbol ester sensitive cell lines (McCoy *et al.*, 1995). Overlapping Sp1 and Egr-1 motifs are found in a number of genes, and in the majority Egr-1 binds to the region with higher affinity than Sp1, thus preventing Sp1 binding (Ebert and Wong, 1995; Khachigian, *et al.*, 1995; Cui *et al.*, 1996). The exact interaction of Egr-1 and Sp1 with the *MDR1* -55 GC box is not yet understood (Cornwell and Smith, 1993a). It has also been demonstrated that the Wilms' tumour suppressor protein, WT-1, which is a member of the Egr family, recognises and binds to the Egr-1 consensus sequences (Harrington *et al.*, 1993; Moshier *et al.*, 1996). In many gene promoters that are activated by Egr proteins, such as Egr-1, Wt-1 has been found to repress transcription (Madden *et al.*, 1993; Madden *et al.*, 1991; Wang *et al.*, 1992). In the *MDR1* promoter WT-1 has been shown to down regulate transcription by binding to the -55GC box. McCoy *et al.*, (1995) found that K562 cells showed activated *MDR1* transcription in response to treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). They then located this TPA-responsive element to the overlapping Sp1/Egr-1 site at the -55GC box. McCoy *et al.*, (1999) later demonstrated that this TPA mediated *MDR1* activation could be inhibited by WT-1 binding to the Sp1/Egr-1 site.

Sp3 is another member of the same family of transcription factors as Sp1 and is able to bind to the same recognition sequence as Sp1. Unlike Sp1, which is predominantly a transcriptional activator, Sp3 can act as either a repressor or an activator (for review see Philipsen and Suske, 1999). In many genes Sp3 can compete for binding with Sp1, thus repressing Sp1 mediated transcriptional activation of that gene. Therefore it is possible

that in the case of *MDR1*, where Sp1 is essential for basal transcription, the relative abundance of the Sp1 and Sp3 may be involved in transcriptional regulation.

Another GC box located at –110 to –103 of the *MDR1* promoter sequence, relative to the transcription start site, is thought to act as a negative response element (NRE) and contain a binding site for a transcription factor that represses *MDR1* transcription. This NRE has been demonstrated experimentally by mutation of the sequence at this region resulting in a promoter activity increasing six fold (Cornwall and Smith, 1993a). Ando *et al.*, (2000), reported that this –110 GC box was methylated in MDR K562/ADM cells and unmethylated in the drug sensitive parental K562 cells, and that demethylation of this site by treating the K562/ADM with DNA methyltransferase inhibitor 5-Aza-2-deoxycytidine decreased P-gp expression in these cells.

A second NRE has been isolated in the sequence between –121 to –111 of the *MDR1* promoter (Ogura *et al.*, 1992). Within this, the region –118 to –113 has been found to contain a CAAT box. Ogretmen and Safa, (1999) used a luciferase reporter gene system to investigate the role of the CAAT box in negative regulation of *MDR1* promoter activity. A wild type 241bp *MDR1* promoter DNA fragment (containing the sequence –198 to +43) or a corresponding mutated 237bp CAAT deleted promoter DNA fragment were cloned into luciferase expressing pGL3-basic vector and transfected into a drug sensitive MCF-7 human breast cancer cell line and its MDR subline MCF-7/Adr. From this it was found that *MDR1* promoter activity was 15 fold higher in MCF-7/Adr cells compared with that of MCF-7. MCF-7 cells transfected with the mutated 237bp CAAT deleted promoter, showed a 12 fold increase in promoter activity compared with MCF-7 cells transfected with the wild type 241bp promoter. However, the MCF-7/Adr cells transfected with the mutated 237bp CAAT deleted promoter showed only a 1.3 fold increase in promoter activity compared to MCF-7/Adr transfected with wild type thus indicating that this CAAT box is involved in downregulation of the *MDR1* promoter in this cell line.

Ogretmen and Safa, (1999) used electrophoretic mobility shift assays (EMSAs) to demonstrate the binding of a protein, later identified as a NF- κ B/p65-cFos hybrid transcription factor, to this –118 to –113 CAAT box in the drug sensitive MCF-7 cells,

but not in the MDR MCF-7/Adr cells. This evidence suggests NF- κ B/p65-cFos produces a negative regulatory effect on the *MDR1* promoter activity through interaction with this region. NF- κ B is a ubiquitous transcription factor that is usually maintained as a dimer in the cytoplasm under the inhibition of I κ B (Bentires *et al.*, 2003). Many chemotherapeutic drugs can induce NF- κ B activity (for review see Pahl, 1999) through stimulating the phosphorylation of I κ B thereby allowing NF- κ B to be localised to the nucleus and induce target genes (Bentires *et al.*, 2003). A study by Bentires *et al.*, (2003) demonstrated that inhibition of NF- κ B resulted in an increase in daunomycin sensitivity and reduced *MDR1* mRNA and P-gp expression in HCT15 human carcinoma cells.

Two c-AMP response sequences (CRS) have been located around -71 and -55 of the *MDR1* promoter sequence. These CRS are identical to the consensus sequences for Sp1 and therefore it is suggested that the interaction of Sp1 with the CRS and the modulation of Sp1 activity by signal transduction through type I cAMP-dependant kinase (PKA) may up-regulate *MDR1* expression (Glazer and Rohlf, 1994, Baggetto, 1997). PKA can phosphorylate Sp1 although it has not been ascertained as to whether phosphorylation of Sp1 effects its binding capabilities. Initial reports of Sp1 hyperphosphorylation, observed in monkey CV1-L cells infected with the simian virus 40 (SV-40), found that the hyperphosphorylation only occurred once Sp1 had bound to its GC box consensus sequence and was facilitated by a DNA-dependent kinase (Jackson, 1990). This study did not observe any change in DNA-binding affinity of the phosphorylated protein or any changes in its transactivating properties (Jackson *et al.*, 1990).

Other studies have observed that phosphorylation altered the DNA binding affinity of Sp1. For example it has been shown that in terminally differentiated liver cells Sp1 becomes phosphorylated which results in downregulation of its DNA binding activity (Leggett *et al.*, 1995). Casein kinase II-mediated phosphorylation of the Sp1 C-terminus region (residues around Thr-579) has also been demonstrated to closely relate to the rate of growth and cell cycle regulation in Balb/c 3T3 cells (Black, *et al.*, 1999; Armstrong *et al.*, 1997).

In the case of *MDR1*, it is thought that hyperphosphorylated Sp1 inhibits phosphoprotein phosphatases, that dephosphorylate the transcription factor CREB. Phosphorylated CREB binds to a c-AMP regulatory element (CRE), found upstream from the CRS (located upstream of the transcription start site) in the *MDR1* promoter, and upon binding *MDR1*, transcription is increased (Baggetto, 1997; Rohlf *et al.*, 1993). In addition to the two CRS there are numerous other consensus binding sites for transcription factors that are controlled by protein kinases, for example, two AP-2 consensus sites are located downstream of the transcription start site at +136 and +382, and an AP-1 consensus site is apparent around –233 from the transcription start site.

Reporter gene studies, by Uchiumi *et al.*, (1993a), using a CAT reporter gene fused to the *MDR1* promoter revealed two stress response elements (SRE) located at –136 to –76 and +1 to +121. By exposing human cancer KB cells transfected with the CAT-*MDR1* reporter gene constructs to ultraviolet irradiation (UV) it was found that an increase in CAT expression, and hence *MDR1* promoter activity, occurred. Similarly, an increase in *MDR1* promoter directed CAT expression also occurred in transfected KB cells in response to cytotoxic drug exposure of the cells to drugs such as 5-fluorouracil (Uchiumi *et al.*, 1993b). Furthermore, the increase *MDR1* promoter activity seen after UV irradiation or exposure to cytotoxic drugs was inhibited in the presence of a protein kinase C (PKC) inhibitor, thus suggesting that phosphorylation of transcription factors by PKC may play a role in the *MDR1* promoter activation in these cells (Uchiumi *et al.*, 1993b). This is supported by a recent study by Ratnasinghe *et al.*, (2001). Here up-regulation of PKC and AP1 subunits c-Jun and c-fos were seen in an MCF-7 drug resistant breast cancer cell line that over expressed *MDR1*, thus suggesting that increased levels of PKC and the transcription factor c-Jun induces *MDR1* up regulation.

Through a series of *MDR1* promoter deletions it was found that the removal of the sequence between –136 to –76 upstream of the transcription initiation site resulted in a reduction in the ability of UV to increase CAT expression, thus it was suggested that this region of the *MDR1* promoter contains a SRE that can activate the *MDR1* promoter (Uchiumi *et al.*, 1993a). In later experiments it was suggested that contained within this –136 to –76 SRE is an inverted CCAAT box, also known as a Y-box, which is situated between –93 to –68 of this sequence (Asakuno *et al.*, 1994). Experiments investigating the importance of the Y-box in *MDR1* promoter regulation have been performed using

the CAT:*MDR1* reporter gene system containing various Y-box directed deletions or mutations. One of these studies reported that deletion of the *MDR1* promoter sequence containing the Y-box showed wild type levels of promoter activity on KB cells (Cornwell, 1993) indicating the Y-box sequence is not essential for normal *MDR1* transcription in these cell lines. This however does not imply that the Y-box does not play a role in induction of *MDR1* expression in response to stress (Asakuno *et al.*, 1994; Ohga *et al.*, 1996; Ohga *et al.*, 1998).

EMSAs on the Y-box from the *MDR1* promoter using nuclear proteins isolated from actinomycin D treated KB cells showed that this Y-box specifically bound a protein which was identified as the transcription factor YB-1 (Asakuno *et al.*, 1994). The binding of YB-1 to a Y-box has been shown to be involved in the regulation of gene expression in several eukaryotic genes, for example the human major histocompatibility complex class II genes (Makino *et al.*, 1996; Swamynathan *et al.*, 1998). Additional experiments on YB-1 have supported the possibility that it may have a role in the stress induction of the *MDR1* promoter activity in response to environmental stimuli such as anti-cancer agents, UV radiation, heat shock, and serum deprivation (Ohga *et al.*, 1996, Ohga *et al.*, 1998). For example, in a study by Asakuno *et al.*, (1994) KB cells demonstrated a 5 fold increase in YB-1 mRNA levels after a 24hour exposure to actinomycin D. Furthermore, Ohga *et al.*, (1998) demonstrated that the binding of YB-1 to the Y-box is directly involved in genotoxic stress activation of the *MDR1* promoter in KB cells through transfection of KB cells containing various *MDR1*:CAT driven constructs with a YB-1 antisense expression plasmid. Transfection of the KB cells with the antisense YB-1 resulted in a decrease in cell YB-1 levels and in decreased binding of YB-1 to the Y-box in EMSAs.

Ohga *et al.*, (1998) also demonstrated that an increase in *MDR1* promoter activity previously seen upon exposure of the KB cells to cytotoxic drugs or UV irradiation (Asakuno *et al.*, 1994; Uchiumi *et al.*, 1993) was decreased by transfection with antisense YB-1. However no effect on promoter activity was seen on transfection with antisense YB-1 in KB cells that had the Y-box deleted from the *MDR1* promoter (Ohga *et al.*, 1998). Therefore this suggests that interaction of YB-1 with the Y-box in KB cells is important for genotoxic stress induction of *MDR1* but not essential for basal

promoter activity. Moreover, Ohga *et al.*, (1996) showed that YB-1 mRNA levels increase following genotoxic stress with agents such as cisplatin and UV light.

The intracellular localisation of the transcription factor YB-1 has also been shown to correlate with MDR. For example a study by Bargou *et al.*, (1997) found that in drug sensitive MCF-7 cells, YB-1 was localised in the nucleus. In addition, transfection of normal P-gp negative breast epithelial HBL-100 cells with YB-1 cDNA resulted in an increase in YB-1 levels in the cytoplasm as well as nuclear localisation of the YB-1, whereas HBL-100 cells that had undergone mock transfection only demonstrated endogenous YB-1 in the cytoplasm. The transfected cells also expressed P-gp and demonstrated increased cell viability in the presence of the cytotoxic drug doxorubicin compared to the mock transfected cells which died rapidly upon drug exposure (Bargou *et al.*, 1997). Also, in a clinical study on 27 untreated primary breast cancers, all patients demonstrated YB-1 in the cytoplasm while the normal breast tissue from the same patient showed no detectable YB-1. In 9 out of 27 of these cases, YB-1 was also localised in the nucleus of the breast cancer cells and these same 9 patients also demonstrated high P-gp levels (Bargou *et al.*, 1997). Hence, there is a correlation between nuclear localisation of YB-1 and intrinsic MDR of primary breast cancers.

However, in contrast to these results, other reports have indicated that it is another Y-box binding factor, NF-Y, that binds to the Y-box in the *MDR1* promoter (Hu *et al.*, 2000; Jin & Scotto, 1998; Sundseth *et al.*, 1997) and has a role in the *MDR1* transcriptional activation through genotoxic stress. For example a recent study by Hu *et al.*, (2000) challenged previous work by re-investigating the Y-box binding factor involved in activation of *MDR1* by UV irradiation in KB-3-1 cells. As with the previous studies Hu *et al.*, (2000) found that the CCAAT box was required for activation of *MDR1* transcription by UV light. However unlike the previous work, an anti-NF-Y antibody and an anti-YB-1 antibody was used in EMSAs to identify the Y-box binding protein interacting with a double stranded Y-box probe. Results from this indicated that in fact NF-Y binds to the Y-box and not YB-1. Other studies have also indicated that in tumour cell lines HCT116, HepG2, KB3-1 and Saos2, NF-Y, and not YB-1 binds to the Y-box elements on the *MDR1* promoter (Sundseth *et al.*, 1997). It was also shown that a double point mutation in the Y-box sequence between -89 and -70 reduced activity of the promoter by 5-10 fold. Thus in these cell lines the Y-box appeared to be essential

for maintaining normal *MDR1* activity. Similar results have been found in a previous study on SW620 colon carcinoma cells and 2780 ovarian carcinoma cells, where deletion or mutation of the sequence between –89 and –70 also resulted in a decrease in promoter activity of around 5-10 fold (Goldsmith *et al.*, 1993). Thus the role of the Y-box in *MDR1* regulation, though clearly important, is unclear at the present time. In some cells it appears to be constitutively essential to *MDR1* activity and in others to inducibly mediated up regulation of *MDR1* in response to genotoxic stress.

MDR1 has also been reported to respond to agents that induce cytochrome P450 (Greiner *et al.*, 1999; Schuetz *et al.*, 1996). Cytochrome P450s catalyse oxidative metabolism of many drugs and result in drug elimination (Schuetz *et al.*, 2000). One cytochrome P450 enzyme CYP3A4 is thought to be responsible for degradation of around 50% of all pharmaceutical agents (Synold *et al.*, 2001). Cytochrome P450s are regulated by hormones, for example regulation of CYP3A4 is increased by glucocorticoid hormones (Schuetz *et al.*, 2000). Transcriptional regulation of CYP3A4 has been shown to involve the orphan nuclear receptor, also known as SXR, PXR, PAR, PRR or NR112 (Synold *et al.*, 2001). Many drugs and steroids that activate the orphan nuclear receptor, and hence CYP3A4, are often also substrates for P-gp (Greiner *et al.*, 1999; Schuetz *et al.*, 1996). The antibiotic rifampicin can induce both CYP3A4 and intestinal P-gp. An SXR nuclear response element, located –7852 to –7837 of the *MDR1* promoter, has been identified as essential to *MDR1* induction by rifampicin in the human colon carcinoma LS174T cell line (Geick *et al.*, 2001).

1.4.5 Post transcriptional mechanisms

Another mechanism suggested to cause an increase in P-gp levels is an increase in the translation rate or stability of *MDR1* mRNA. The half life of mRNA can be controlled by many mechanisms, for example deadenylation-dependant mRNA decay where a poly(A)-specific deadenylating nuclease shortens the poly(A) tail which is followed by removal of the methylguanosine cap and 5' and 3' exonucleases degrade the mRNA (reviewed in Bernstein and Ross., 1989; Ross., 1995; Jacobson and Peltz, 1996; Wilusz *et al.*, 2001). Though not yet fully understood, it is thought that interaction with a

poly(A)-binding protein(PABP) can inhibit mRNA degradation thus allowing stabilisation of the mRNA and promoting translation (Wilusz *et al.*, 2001).

A study comparing levels of MDR, P-gp expression and *MDR1* mRNA levels for different cell lines found that when selecting for a high level of vincristine resistance in SK VCR 2 cells (a MDR subline of the SKOV3 human ovarian carcinoma cell line) an increase in MDR and P-gp expression was seen without a proportionate increase in *MDR1* mRNA or gene amplification. This indicated that P-gp expression was regulated translationally and not transcriptionally (Bradley *et al.*, 1989). A further study by Marino *et al.*, (1990), showed that regenerating rat liver, which over expresses P-gp, shows a 20 fold increase in the level of *MDR1* mRNA compared to normal rat liver, which expresses P-gp at physiological levels. However nuclear run-on experiments, which allow the direct measurement of the rate of transcription of a specific gene, indicated there was no significant differences between the rate of *MDR1* transcription in regenerating rat liver compared to normal liver cells (Marino *et al.*, 1990). These results suggest that *MDR1* overexpression can be directly linked to increases in mRNA stability.

1.5 The current study

In this project in order to investigate molecular mechanisms of regulation of *MDR1* in drug resistant cancer cells, K562, a human leukaemic cell line and K562 derived MDR sublines were used as an *in vitro* model of acquired MDR. K562 is a cell line derived from a patient with chronic myeloid leukaemia and is relatively sensitive to chemotherapeutic drugs (Lozzio & Lozzio, 1979). P-gp expression in K562 cells is generally considered to be negative (Marks *et al.*, 1993; Marks *et al.*, 1996) although some investigators believe that these cells have a very low level of expression (Hamada & Tsurdo, 1986, Yague *et al.*, 2003). Several MDR sublines have previously been isolated by exposure of K562 cells to clinically relevant levels of drugs such as epirubicin (Marks *et al.*, 1993) and daunorubicin (Hargrave *et al.*, 1995). These MDR sublines express P-gp and the drug resistance is reversed by modulators of P-gp

function, indicating that activation of P-gp expression is the cause of the MDR in these cell lines.

The cells lines used in this study exhibit relatively low levels of MDR mediated by P-gp, however, each cell line was chosen so as to cover a range of resistance levels, that is from low to high, within this low level MDR. The K/E15B cell line was derived from parental K562 cell lines by multiple exposures to 10ng/ml of epirubicin for 3-14 days over a 3 month period, then in turn exposing these cells to 15ng/ml of epirubicin for 3-14 days over a further 3 month period (Marks *et al.*, 1993). K/E15B cells were cloned by limiting dilution to obtain the K/EC32 subclone (Ishri, Honours thesis, 1997). The K/EC32 cell line demonstrates a very low level of MDR. K/EPR and K/DNR cells were developed by Hargrave *et al.*, (1995), by intermittently exposing K562 cells to either 20ng/ml of epirubicin (K/EPR) or 20ng/ml of daunorubicin (K/DNR) for 3 days over a 2 month period. The K/EPR cell line was chosen to represent an intermediate level of MDR and the K/DNR represents a higher level of MDR. All of these K562 MDR sublines demonstrate a classical MDR phenotype and through flow cytometry and Western blotting it is also known that these cells express P-gp (Hargrave *et al.*, 1995; Marks *et al.*, 1993).

This project investigated the possible mechanisms for the activation of P-gp expression in the development of the K562 MDR cell lines from the sensitive K562 parental cells. Some *in vitro* cell lines in the literature require re-exposure to drug at regular intervals or constant exposure to low concentrations of drug in order to maintain MDR levels (Tohda *et al.*, 1997; Hu *et al.*, 1995). Others however, are able to maintain stable MDR levels (Geroni *et al.*, 1993). Thus the stability of MDR in the K562 derived MDR cell lines and the effect of drug re-exposure on MDR levels was also investigated.

CHAPTER 2

Characterisation of experimental cell lines

2.1 INTRODUCTION

To confirm drug resistance levels of the K562 MDR sublines used in this study, cytotoxicity assays with the drugs vinblastine and paclitaxel were performed. Previously reported Epirubicin resistance levels for the K562 MDR cell lines, relative to the parental K562 cells, were 1.7 fold for K/EC32 (Ishri, Honours thesis, 1997), 4-fold for K/EPR (Hargrave *et al.*, (1995) and 6.8 fold for K/DNR (Hargrave *et al.*, (1995). *MDR1* mRNA levels were also determined by Northern blot analysis in order to see if the relative quantity of *MDR1* mRNA in the cells, correlated to their level of MDR. RNA isolated from the VLB100 cell line was also used in Northern blots as a positive control as these cells contain amplified copies of the *MDR1* gene and express large amounts of *MDR1* mRNA (Hill *et al.*, 1988). Gene amplification can occur *in vitro* during the selection of multidrug resistant cell lines when the cells are exposed to high concentrations of drugs (Shen *et al.*, 1986) and so to exclude *MDR1* gene amplification as the cause of differing resistance levels in the K562 MDR sublines, Southern slot blot analyses were also performed.

To investigate the stability of MDR in the K562 MDR sublines, cells were grown for varying periods of time in the absence of drug, then MDR levels re-examined. Inducibility of MDR was also tested by determining the effect of single short-term exposure of the MDR cell lines to cytotoxic drugs for varying lengths of time.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines and culture

The K562 cell line was obtained from the ATCC culture collection. The K562 cell line was obtained from the ATCC culture collection. It was originally derived from a patient with chronic myeloid leukaemia and is relatively sensitive to chemotherapeutic drugs (Lozzio & Lozzio, 1979). The K562 drug resistant subline K/E15B was derived from parental K562 cell lines by multiple exposures to 10ng/ml of epirubicin for 3-14 days over a 3 month period, then in turn exposing these cells to 15ng/ml of epirubicin for 3-14 days over a further 3 month period (Marks *et al.*, 1993). The K/EC32 cell line is a subclone of K/E15B isolated by cloning K/E15B cells by limiting dilution (Ishri, Honours thesis, 1997). The K/EPR and K/DNR cell lines were developed by Hargrave *et al.*, (1995), by intermittently exposing K562 cells to either 20ng/ml of epirubicin (K/EPR) or 20ng/ml of daunorubicin (K/DNR) for 3 days over a 2month period. CEM/VLB100 cells (VLB100) is a multidrug resistant subline originally developed from human leukaemia cell line CCRF-CEM (Beck *et al.*, 1979).

Cells were cultured in RPMI-1640 medium supplemented with 20mM HEPES, 10% foetal calf serum and NaHCO_3 (0.85g/L) at 37°C with 5% CO_2 tension in a humidified incubator. The cells were maintained by passaging every 2-3 days.

2.2.2 Cytotoxicity Assays

Exponentially growing cells (4×10^4 cells per well) were placed in 100 μL aliquots into 96 well microtitre plates. 100 μL of 2-fold serially diluted paclitaxel (Sigma-Aldrich) or vinblastine (David Bull Laboratories, Australia) was then added to cells in triplicate wells. The plates were incubated for 4 days at 37°C with 5% CO_2 tension in a humidified incubator, after which cell viability was assessed via an MTT assay (Marks

et al., 1992). Control wells were treated as above except that 100 μ l of RPMI-1640 media was used in place of drug dilution. For reversal of drug resistance, 10mM verapamil (Sigma, USA) was added to the cells in the assay. The mean cell viability and standard deviation of triplicate wells was determined and plotted against drug concentration using Graph Pad Prism Version 3. Relative resistances were determined by dividing the drug concentration giving 50% inhibition of growth of the MDR cell line with that giving 50% inhibition of growth of the drug sensitive K562 cells determined in the same experiment.

2.2.3 Total RNA Extraction

Total RNA was isolated from 1×10^7 exponentially growing cells using a method adapted from Chomczynski and Sacchi (1987). Cells were washed in 0.1M ice cold sterile PBS and centrifuged at 500xg for 5 minutes at 4°C. The cells were lysed in 1ml a solution of 4M guanidinium isothiocyanate, 2.5mM sodium citrate pH7.0 at RT, 0.5% sarcosyl, 0.1M β -mercaptoethanol. Genomic DNA was sheared by dispelling the cell lysate at least 10x through a sterile 23 gauge needle then 0.1ml of 2M sodium acetate (pH 4.0), 1.0ml of water saturated phenol and 0.2ml of chloroform:isoamyl alcohol (49:1) was sequentially added, with mixing by inversion after each addition. This was then shaken vigorously for 10 seconds, incubated on ice for 15 minutes and centrifuged at 10, 000xg for 20 minutes at 4°C. The upper aqueous phase containing the RNA was collected and mixed with an equal volume of chloroform:isoamyl alcohol (49:1) then vigorously shaken for 10 seconds, incubated on ice for 15 minutes and centrifuged at 10, 000xg for 20 minutes at 4°C. From this the aqueous phase was collected, 1ml of isopropanol added and following incubation at -20°C for at least 1 hour RNA was collected by centrifugation at 10,000xg for 20 minutes at 4°C and the pellet resuspended in 300 μ l of solution of 4M guanidinium isothiocyanate, 2.5mM sodium citrate pH7.0 at RT, 0.5% sarcosyl, 0.1M β -mercaptoethanol. 300 μ l of isopropanol was then added and following incubation at -20°C for 1 hour, the samples were spun at 10,000xg for 20 min at 4°C and the RNA pellets washed in 70% ethanol and centrifuged at 10, 000xg for 10 minutes at 4°C. RNA pellets were air dried and resuspended in 50 μ l of sterile diethylene pyrocarbonate treated water and stored at -80°C. The concentration of the RNA was

determined by reading the absorbance of the samples at 260nm and 280nm. Calculation of the RNA concentration was performed as follows:

$$\text{RNA concentration} = A_{260\text{nm}} \times 40\mu\text{g/ml} \times \text{dilution factor.}$$

Where A_{260} is the absorbance at 260nm and 40 $\mu\text{g/ml}$ is the RNA concentration equivalent to 1 absorbance unit.

2.2.4 RNA Transfer and Northern Hybridisation

20 μg of total RNA was electrophoresed on 1% Agarose gels in 1x MOPS (0.04 M Morpholinopropanesulfonic acid (free acid); 0.01 M Na-acetate-3 x H₂O; 1mM EDTA; adjusted to pH 7.2 with NaOH) and 2% formaldehyde, then vacuum transferred onto Hybond N+ nylon membranes (Amersham life Sciences, Australia) for 1 hour in 1x SSC (0.15 M NaCl plus 0.015 M sodium citrate), 10mM NaOH. RNA was UV cross linked to membranes at 70,000 $\mu\text{J}/\text{cm}^2$ in a UV cross-linker (Stratalinker, Australia). Membranes were then hybridised with a ³²P- labelled *MDR1* cDNA probe. The *MDR1* cDNA probe was prepared by EcoRI digestion of the *MDR1* cDNA containing plasmid pMDR2000XS (Pastan *et al.*, 1988) and isolating a 3.4kb cDNA fragment on a 1.5% agarose gel run in 1X TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0) running buffer along with pGEM DNA markers. The 3.4kb band was excised from the gel and purified by the GeneClean method (GeneWork, Australia). Typically, 25-50ng of the probe was random primer labelled with 50 μCi of α -³²P-dCTP (3000 $\mu\text{Ci}/\text{mmol}$) using the Gigaprime DNA labelling kit (GeneWork, Australia) according to the supplied protocol. Unincorporated nucleotides were removed by column chromatography with Sephadex G50.

Membranes were prehybridised at 50°C for at least 1 hour in NorthernMax pre-hybridisation/hybridisation solution (Ambion, USA). The solution was then replaced with fresh pre-hybridisation/hybridisation solution to which the pMDR2000xs EcoRI fragment probe (10ng/ml, denaturated by addition of 1/9 volumes of 4M NaOH and incubating for 15 min at RT) was added and membranes were hybridised overnight at 50°C. Membranes were washed 2 times in NorthernMax wash solution #1 (Ambion, USA) for 5-10 min at RT, followed by 2, 15-20 min washes in NorthernMax wash

solution #2 (Ambion, USA) at 50°C, then exposed to a BioRad phosphorimager (BI) screen and visualised using a BioRad phosphorimager. Membranes were subsequently stripped in boiling 0.1% SDS and re-probed with a 0.5µg *GAPDH* RNA probe. The *GAPDH* was *in vitro* transcribed from a linearised mouse DNA template (Ambion, USA) using a MAXIscript *in vitro* transcription kit (Ambion, USA) and labelled with 10µCi, 3000Ci/mmol of α -³²P-dUTP according to manufacturer's instructions. Membranes were prehybridised, hybridised and washed as for the pMDR2000xs EcoRI fragment probe except 65°C prehybridisation, hybridisation and stringent wash temperature was used.

2.2.5 Isolation of Genomic DNA

Genomic DNA was isolated from the K562 cell lines and each MDR sublines. Briefly, 1×10^7 cells were pelleted at 500xg for 5 min and resuspended in 9ml of ice cold TLB (0.32M sucrose, 5mM MgCl₂, 10mM Tris pH7.6 at RT, 1% Triton X100). Nuclei were collected by centrifugation at 10,000xg for 5 min at 4°C. The pellet was evenly resuspended in 450µl of ice cold TEN (0.1M Tris pH8.0 at RT, 20mM EDTA, 0.15M NaCl) then 25µl of 20% SDS and 25µl of freshly prepared 2mg/ml proteinase K was added with mixing after each addition. This lysate was incubated at 50°C with frequent mixing by inversion for 2 hours, followed by incubation at 37°C overnight. An equal volume of Tris pH 7.0 saturated phenol was then added to the cell lysate, which was emulsified by repeated gentle inversions and spun at 10,000xg for 5 min. The upper phase was collected, taking care to avoid the protein containing white flocculent at the interface, and re-extracted with phenol until there was no white band present at the interface. Following this, sequential extractions, firstly with equal volumes of phenol and chloroform, then with an equal volume of chloroform were performed on the upper aqueous phase. To precipitate the DNA, 2 volumes of absolute ethanol were added. The DNA was then spooled on a bent sterile pasteur pipette, washed by dipping in 70% ethanol and air dried before being redissolved in 100µl of sterile dH₂O.

2.2.6 Slot Blot Transfer and Southern hybridisation

10 μ g of genomic DNA was added to a transfer buffer containing 1M NaOH and 10mM EDTA, in a final volume of 500 μ l. The DNA was then denatured by heating at 95°C for 5 min then plunged into ice. The samples were then applied to a PR600 SlotBlot (Hoefer Scientific Instruments, USA) in duplicate and vacuum transferred in 0.4M NaOH onto Hybond N+ nylon membrane (Amersham Life Sciences, Australia) according to manufacturer's instructions. Slot blot membranes were prehybridised at 65°C for at least 1 hour in 6.5ml of hybridisation solution (2x SSPE [0.36 M NaCl, 20 mM NaPO₄ (pH 7.7), 1 mM EDTA], 1% SDS, 6.5 μ g BSA and 320 μ g of sonicated fish testes DNA). After pre-hybridisation this solution was removed and replaced with fresh hybridisation solution to which 10ng/ml of labelled *MDR1* cDNA probe (prepared as previously described in section 2.2.4) was added along with an amount of glacial acetic acid equivalent to the amount of NaOH added previously. After hybridisation at 65°C overnight the membrane was then washed sequentially with a 2x SSC rinse at RT, 0.5x SSC and 0.1% SDS for 15 min with agitation at RT, 0.5x SSC and 1% SDS for 15 min with agitation at 65°C and finally rinsed in 0.5x SSC and 0.1% SDS before exposure to a BioRad phosphoimager (BI) screen and visualised using a BioRad phosphoimager. Image analysis was performed using the Molecular Analyst Program (BioRad, Australia).

After exposure, the membrane was stripped in boiling 0.1% SDS then re-probed with a *GAPDH* RNA probe (as previously described in section 2.2.4). Membranes were pre-hybridised at 50°C for at least 1 hour in NorthernMax pre-hybridisation/hybridisation solution (Ambion, USA). This solution was then replaced with fresh pre-hybridisation/hybridisation solution to which the *GAPDH* probe was added and membranes were hybridised overnight at 50°C. The membrane was washed 2 times in NorthernMax wash solution #1 (Ambion, USA) for 5-10 min at RT, followed by 2, 15-20 min washes in NorthernMax wash solution #2 (Ambion, USA) at 50°C. Membranes were exposed to a BioRad phosphoimager (BI) screen and visualised using a BioRad phosphoimager. Image analysis was performed using the Molecular Analyst Program (BioRad, Australia).

2.2.7 Drug induction

2.2.7.1 3 day drug treatments

Cells at an initial density of $0.7 \times 10^6/\text{ml}$ were treated with epirubicin (David Bull Laboratories, Australia) at a concentration of 5ng/ml for K562, 15ng/ml for K/E15B subclone K/EC32 and 20ng/ml for K/EPR cells. K/DNR cells were treated with 20ng/ml daunorubicin (David Bull Laboratories, Australia). The cells were exposed to drug for 3 days at 37°C with 5% CO₂ tension in a humidified incubator, then allowed to recover by culture in drug free media for 2 weeks before cytotoxicity assays were performed.

2.2.7.2 4 and 16 hour drug treatments

Four and 16 hour drug treatments were performed on the K/EPR cells using epirubicin, paclitaxel, cisplatin and rifampacin. K/EPR cells at an initial density of $0.7 \times 10^6/\text{ml}$ were treated with epirubicin (David Bull Laboratories, Australia) at a concentration of 160ng/ml, paclitaxel (Sigma-Aldrich, Australia) at 50ng/ml, cisplatin (David Bull Laboratories, Australia) at 500ng/ml or with rifampacin at 10 μM , 30 μM and 100 μM . The cells were exposed to drug for 4 or 16 hours at 37°C with 5% CO₂ tension in a humidified incubator, then allowed to recover by culture in drug free media for 3 days before cytotoxicity assays were performed.

The concentration of epirubicin, paclitaxel and cisplatin chosen for the 4 and 16 hour drug treatments were selected on the basis of their approximate equivalence to an IC₂₅ caused by exposure for these times. To determine these concentrations, exponentially growing cells (4×10^4 cells per well) were placed in 100 μL aliquots into 96 well microtitre plates and 100 μl of 2-fold serially diluted drug was then added to the cells in triplicate wells. The plates were incubated for 4 or 16 hours at 37°C with 5% CO₂ tension in a humidified incubator, after which cell viability was assessed via an MTT assay (Marks *et al.*, 1992). Control wells were treated as above except that 100 μl of RPMI-1640 media was used in place of drug dilution. The IC₂₅ caused by either a 4 or 16 hour drug exposure was similar. A range of concentrations of rifampacin was used for the 4 and 16 hour treatments.

2.2.8 Trichostatin A treatment of cells

Exponentially growing K562 and K/EPR cells were incubated with a range of Trichostatin A (TSA) (Sigma-Aldrich) concentrations (0, 50, 200, 500, 1000nM) for 16 hours at 37°C with 5% CO₂ tension in a humidified incubator. As TSA was dissolved in ethanol, cell growth was assessed and ethanol controls were carried out for all assays. After TSA exposure, cells were washed in RPMI and used in an epirubicin cytotoxicity assay as previously described in section 2.2.2. The effect of TSA on cell growth was determined by performing cell counts on cells exposed to the various TSA concentrations for 16 hours, then allowed to recover for three days in drug free medium.

2.3 RESULTS

2.3.1 Drug Resistance Levels of K562 MDR Cell Lines

To confirm MDR levels of the model cell lines, cytotoxicity assays were performed on K562, K/EC32, K/EPR, K/DNR and the parental K562 cells using either of the chemotherapeutic drugs vinblastine or paclitaxel. To confirm that the mechanism of drug resistance in the K562 MDR cell lines was P-gp mediated, cytotoxicity assays were performed on each of the cell lines in the presence and absence of the chemosensitising agent verapamil. As well as verapamil, paclitaxel itself is a good indicator of P-gp mediated MDR as it is not a substrate for multidrug resistance protein (MRP), the other main efflux means for cytotoxic drugs (Grant et al., 1994).

Figures 2.1(a) and (b) respectively show results for a vinblastine and a paclitaxel cytotoxicity assay for the K/EC32 subline. The results indicate that K/EC32 cells were 2.4 fold resistant to vinblastine (Figure 2.1(a)) and 3.9 fold resistant to paclitaxel (Figure 2.1(b)) relative to the parental K562 cell line. These assays were performed at

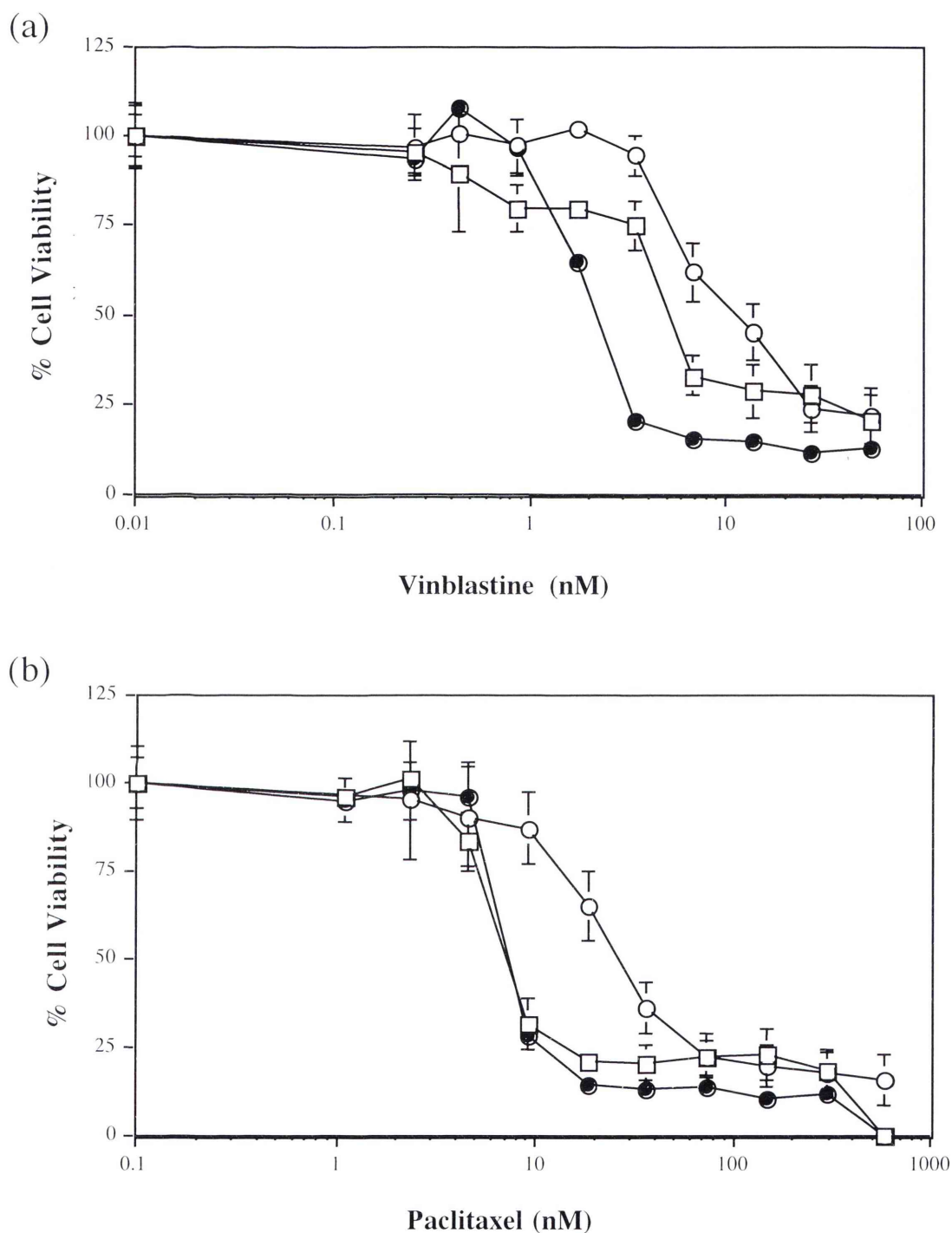


Figure 2.1 Resistance levels of K/EC32 cells. The K/EC32 (○) cell line was assayed for its resistance to (a) vinblastine and (b) paclitaxel compared to the resistance of the parental K562 cell line (□). These assays were performed in the presence (●) and absence (○) of the chemosensitising agent verapamil. Points are the mean of triplicate wells and bars are the standard deviation.

least five times with similar results. The resistance demonstrated by the K/EC32 cells was completely reversed in the presence of verapamil. The reversal of K/EC32 resistance with vinblastine was performed three times reproducibly. Vinblastine and paclitaxel cytotoxicity assays were also performed on the K/EPR cell line and the results of one of these assays are shown in Figures 2.2(a) and (b) respectively. In these assays K/EPR cells demonstrated 6.9 fold resistance to vinblastine (Figure 2.2(a)) and an 8.8 fold resistance to paclitaxel (Figure 2.2(b)) relative to the parental K562 cell line. K/EPR vinblastine cytotoxicity assays were performed three times and the paclitaxel cytotoxicity assays five times with similar results. Like the K/EC32 cell line, the resistance of the K/EPR cells was completely reversed in the presence of verapamil. This reversal of K/EPR resistance was repeated in a subsequent experiment.

Figures 2.3(a) and (b) show the corresponding results for one of the cytotoxicity assays carried out on the K/DNR cell line. These results indicate that, relative to K562 cells, the K/DNR cells were 16.7 fold resistant to vinblastine (Figure 2.3(a)) and 21.0 fold resistant to paclitaxel (Figure 2.3(b)). K/DNR vinblastine cytotoxicity assays were performed three times and the paclitaxel cytotoxicity assays five times with similar results. Once again all resistance was reversed in the presence of verapamil. This reversal of K/DNR resistance was repeated in a subsequent experiment.

The results of these cytotoxicity assays indicate that a range of low (K/EC32), medium (K/EPR) and high (K/DNR) state, albeit generally low level MDR cell lines are being used in the current study.

2.3.2 *MDR1* mRNA Levels in K562 and MDR Cell Lines

The cells used in this study were derived from the same K562 parental cell line and yet all exhibit different levels of MDR. In order to determine if the relative quantity of *MDR1* mRNA in the cells correlated to level of MDR displayed by the sublines Northern Blots were performed on total RNA extracted from K562 and MDR sublines at the same time the paclitaxel sensitivity assay was conducted. RNA extracted from the highly *MDR1* expressing VLB100 cell line (Beck *et al.*, 1979) was used as a positive control. After hybridisation with the *MDR1* probe (representative blots shown

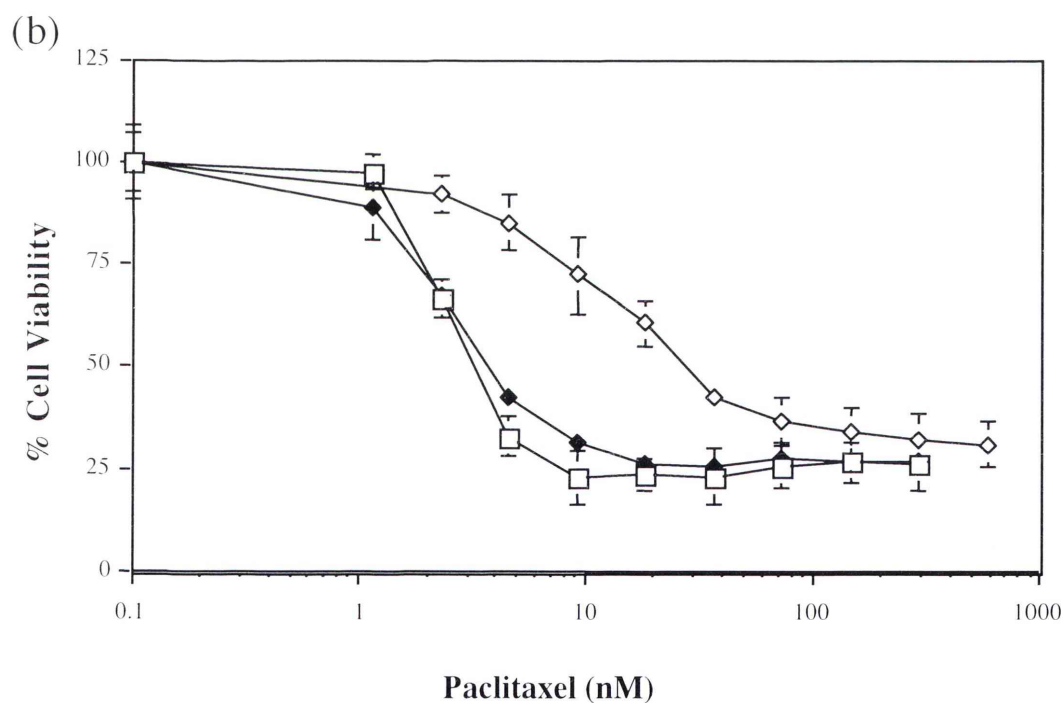
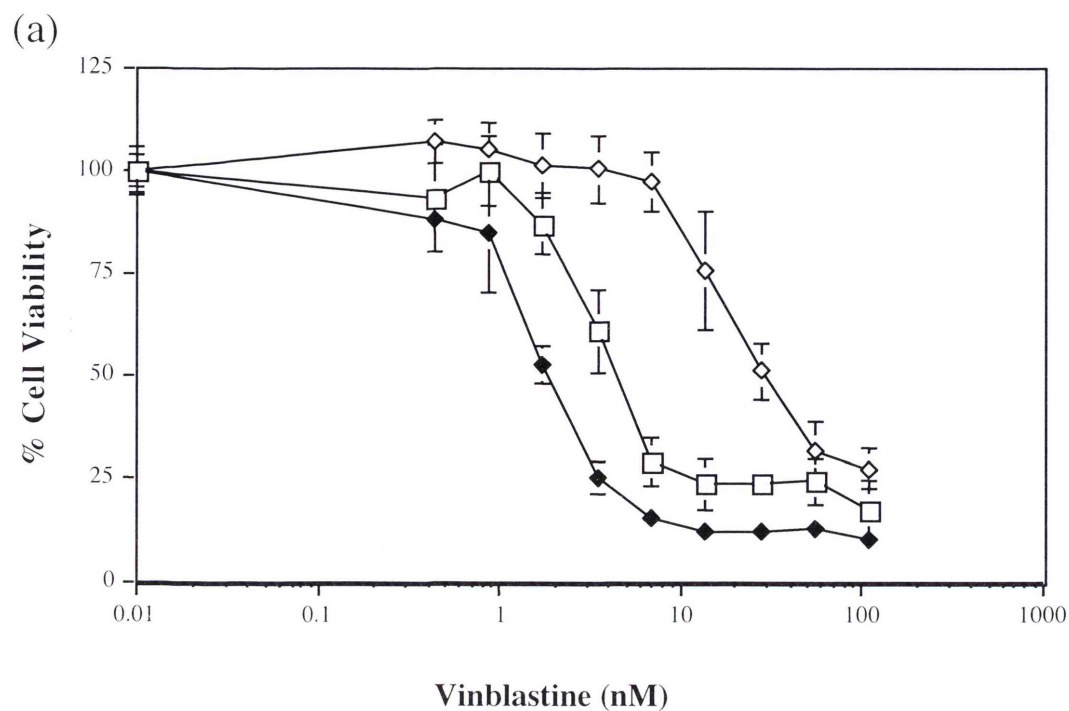


Figure 2.2 Resistance levels of K/EPR cells. The K/EPR (◇) cell line was assayed for its resistance to (a) vinblastine and (b) paclitaxel compared to the resistance of the parental K562 cell line (□). These assays were performed in the presence (◆) and absence (◇) of the chemosensitising agent verapamil. Points are the mean of triplicate wells and bars are the standard deviation.

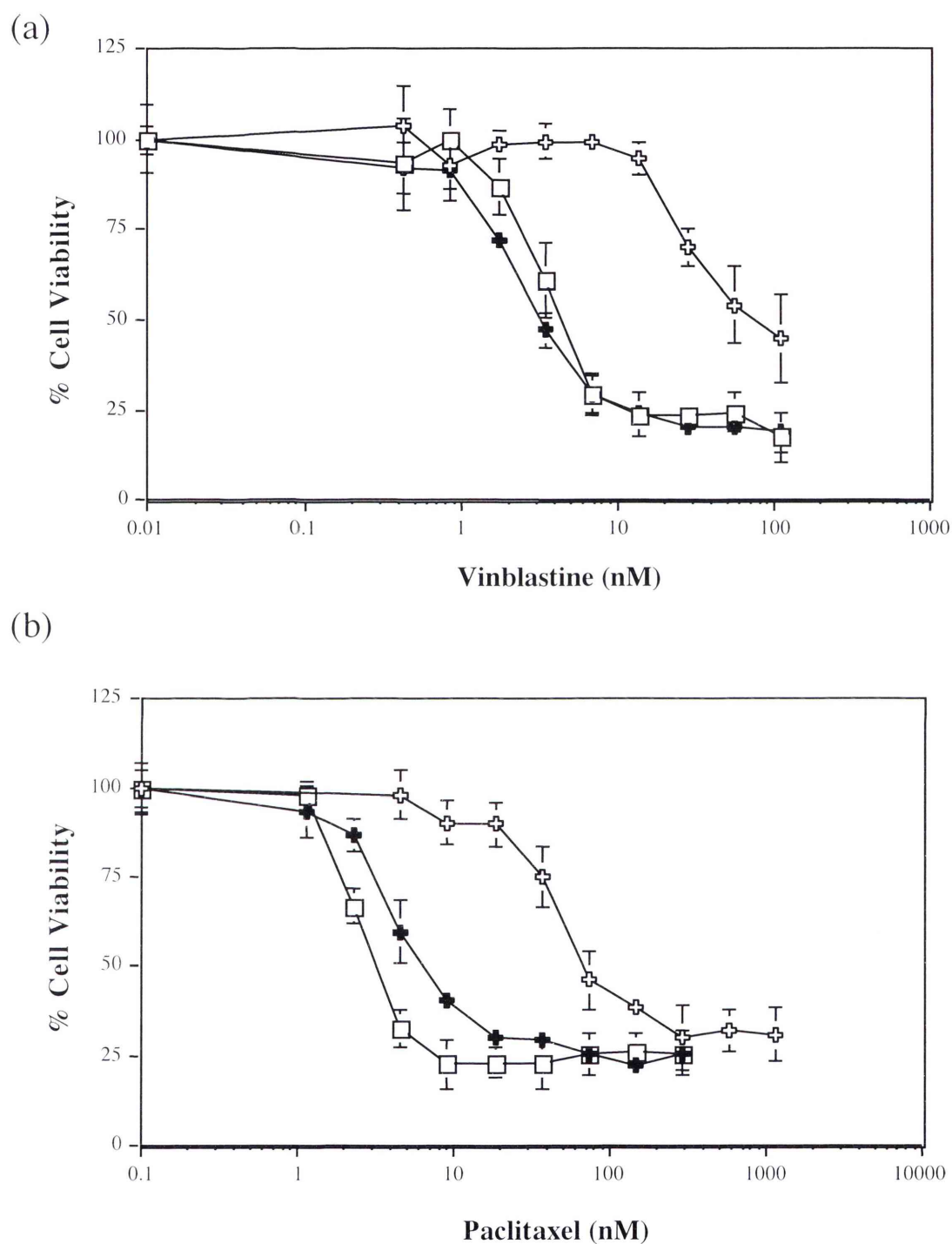


Figure 2.3 Resistance levels of K/DNR cells. The K/DNR (◻) cell line was assayed for their resistance to (a) vinblastine and (b) paclitaxel compared to the resistance of the parental K562 cell line (◻). These assays were performed in the presence (⊕) and absence (◻) of the chemosensitising agent verapamil. Points are the mean of triplicate wells and bars are the standard deviation.

in Figure 2.4a) the membrane was then stripped and re-hybridised with a GAPDH RNA probe to normalise RNA loadings (representative blot shown Figure 2.4b). *MDR1* mRNA was detected in each of the MDR cell lines but not in the drug sensitive K562 cells. Normalised levels of *MDR1* mRNA were determined by performing densitometry using the Molecular Analyst program (BioRad, Australia). Table 2.1 shows the normalised levels of *MDR1* mRNA in the cell lines and also correlates these levels with the levels of paclitaxel relative resistance determined at the time the RNA was extracted. Examination of this data shows a positive relationship between the level of *MDR1* mRNA present in the cells and the levels of MDR displayed by the cells at that time. These results were reproduced three times with similar results.

2.3.3 *MDR1* gene copy number

To investigate the possibility that Pgp up-regulation was due to amplification of the *MDR1* gene copy number genomic DNA was extracted from each cell lines and used in slot blot Southern hybridisation with a ³²P-labelled pMDR2000XS EcoRI fragment

DNA probe (refer to section 2.2.4) (Figure 2.5(a)). The amount of DNA loaded on the membrane was normalised for each cell line by stripping then re-probing the membrane with a ³²P- labelled *GAPDH* RNA probe (Figure 2.5(b)). From these results and the densitometry results shown in Table 2.2, it is evident that each of the cell lines contains equivalent copy numbers of the *MDR1* gene, which in turn is equivalent to the number of copies present in the parental K562 cell line. This results was reproduced in a subsequent experiment. Therefore in the K562 derived MDR sublines *MDR1* upregulation is due to a factor other than *MDR1* gene amplification.

2.3.4 Stability of MDR in K562 MDR sublines

The stability of MDR was investigated by repeated cytotoxicity determination over an extended period of time in drug free culture following establishment from frozen stocks. Figure 2.6 shows a summary of the paclitaxel resistance levels (relative to K562) of the MDR sublines determined after various periods of time in drug free culture.

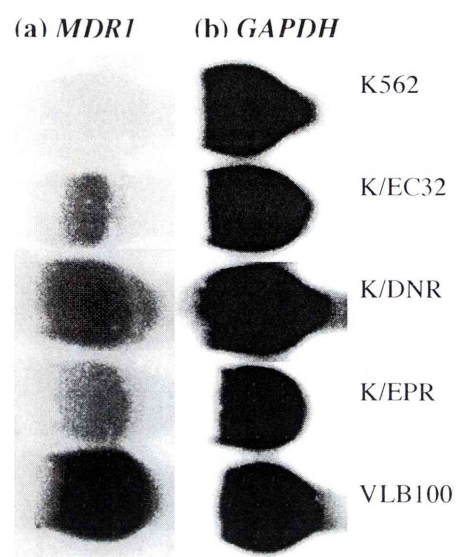


Figure 2.4 Northern blot for the determination of *MDR1* mRNA levels in K562 MDR cell lines. Total RNA from K562, and MDR sublines before, and after drug treatment, was hybridised with (a) an *MDR1* DNA probe, before stripping and re-hybridisation with (b) a *GAPDH* RNA probe. VLB 100 RNA was used as a positive control.

Table 2.1 Normalised *MDR1* mRNA levels in K562 MDR cell lines and their corresponding relative resistances to vinblastine and paclitaxel.

| Cell Subline | Normalised <i>MDR1</i> Level * | Relative Vinblastine Resistance # | Relative Paclitaxel Resistance # |
|--------------|--------------------------------|-----------------------------------|----------------------------------|
| K/EC32 | 2.5 | 2.4 | 3.9 |
| K/EPR | 3.6 | 6.9 | 8.8 |
| K/DNR | 6.6 | 16.7 | 21.0 |

* *MDR1* mRNA levels normalised to *GAPDH* mRNA levels.

resistance levels shown are relative to that of untreated K562 cells and refer to the individual IC₅₀ drug concentrations.

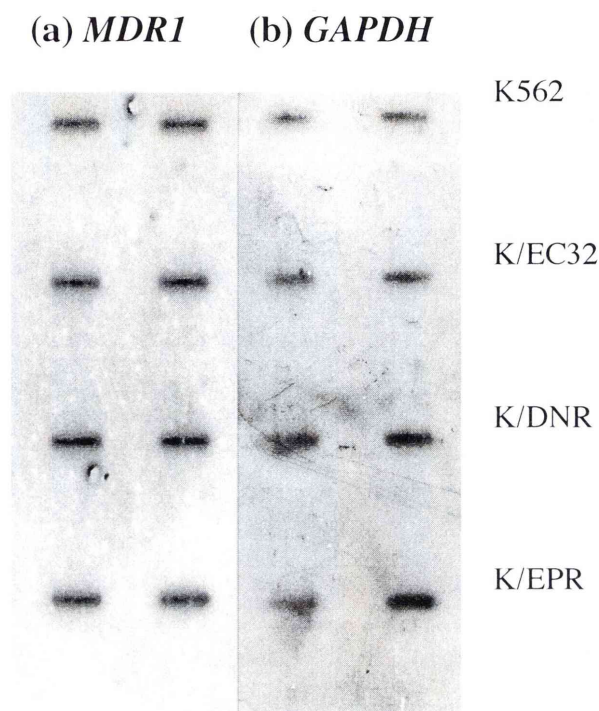


Figure 2.5 Comparison of the *MDR1* gene copy number in K562 MDR cell lines. DNA from K562, K/EC32, K/DNR and K/EPR was used in duplicate in a slot blot Southern Hybridisation with (a) an *MDR1* DNA probe before stripping and re-hybridising with (b) a *GAPDH* RNA probe to normalise loadings.

Table 2.2 *MDR1* gene copy number normalised to *GAPDH* gene copy numbers in K562 MDR cell lines

| | K562 | K/EC32 | K/DNR | K/EPR |
|---|------|--------|-------|-------|
| Normalised¹ level | 1.17 | 1.08 | 1.00 | 0.92 |

¹ shows the average of duplicate *MDR1* slot blot intensities divided by average of duplicate *GAPDH* slot blot intensities

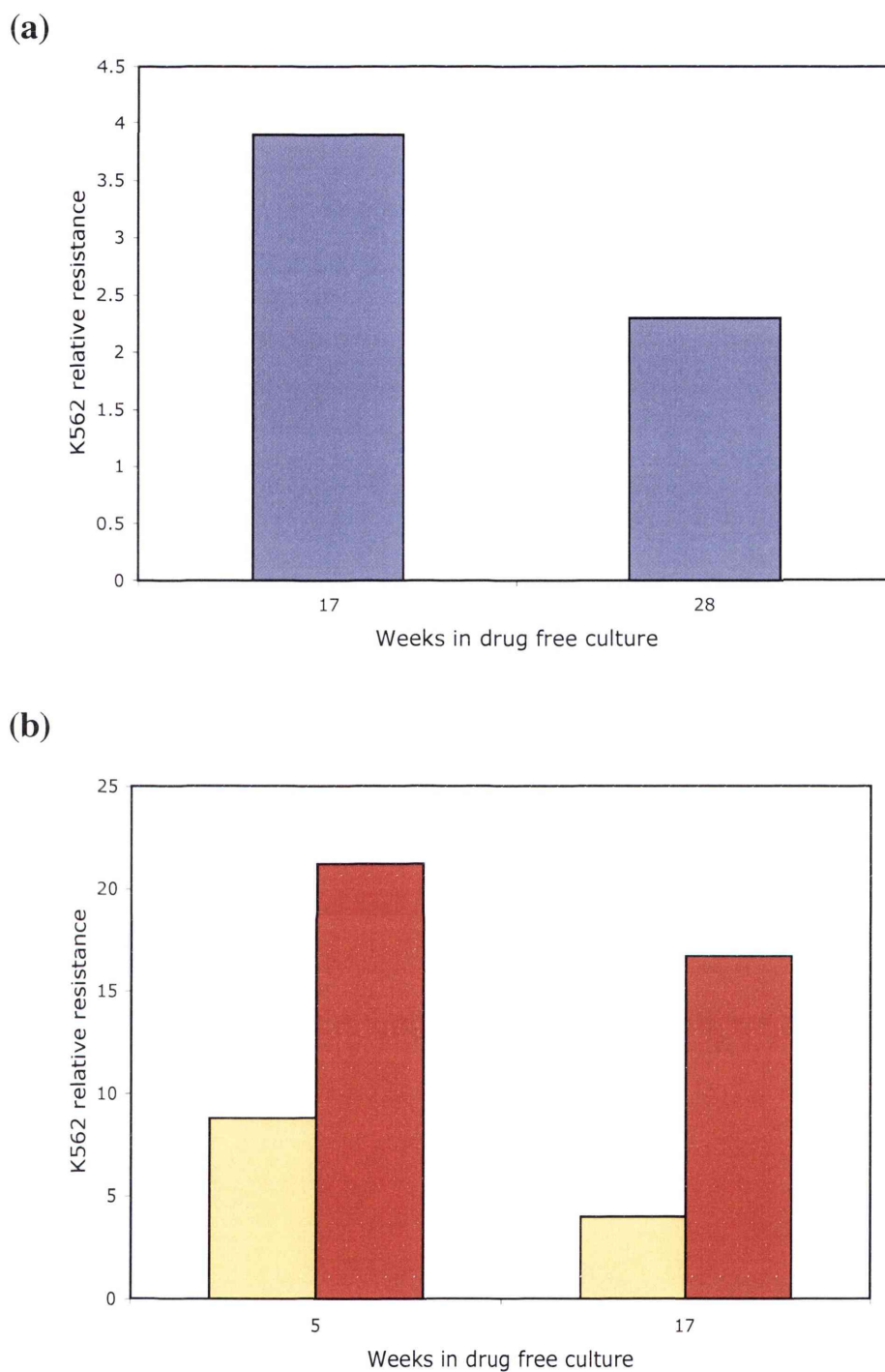


Figure 2.6 Stability of MDR. (a) Relative paclitaxel resistance of K/EC32 after culture for 17 or 28 weeks in the absence of drug (■) (b) Relative paclitaxel resistance of K/EPR (■) and K/DNR (■) cells were determined after being maintained for 5 or 17 weeks in drug free culture.

Examination of this data indicates that the resistance levels of the non-drug treated cell lines decreases with time in culture. From Figure 2.6a it can be seen that K/EC32 cells maintained for 17 weeks in drug free culture were 3.9-fold resistant to paclitaxel relative to K562, but after 28 weeks of drug free culture paclitaxel resistance in K/EC32 cells had decreased to 2.3-fold. This assay was repeated six times with similar results.

Figure 2.6b shows the corresponding results for the K/EPR and K/DNR sublines. As was seen in the K/EC32 cells, the K/EPR and K/DNR sublines also demonstrated decreased resistance with culture in drug free media. The K/EPR decreased from an 8.8 fold resistance (relative to K562) after 5 weeks in drug free culture to 4.0 fold after 17 weeks. Similarly, K/DNR showed a 21.2 fold resistance at week 5, decreasing to 16.7 fold after 17 weeks in drug free culture. These assays were performed three times with similar results.

2.3.5 Effect of 3 Day Re-exposure to Drug

As the K562 MDR sublines did not appear to be stable in long term drug free culture, cells were re-exposed to drugs to determine if MDR levels could be re-stimulated. Cells were treated for 3 days with the original selecting drug concentration and then cytotoxicity to paclitaxel was assessed following a short period of recovery. The drug concentration used typically produces less than 50% inhibition of cell viability in a standard 4 day cytotoxicity assay (data not shown).

Figure 2.7 shows the result obtained following a 3 day exposure to 15ng/ml of epirubicin of the K/EC32 subline after 17 weeks in drug free medium. The untreated control cells K/EC32 and drug treated cells, designated K/EC32T, were found to be 3.9-fold and 14.1-fold respectively, more resistant to paclitaxel than the K562 cells (cytotoxicity assays were performed six times with similar results). Thus, short term drug exposure of the K/EC32 subclone was able to increase the level of drug resistance displayed by these cells. The induction of drug resistance by 3 day epirubicin exposure of the K/EC32 cell line was also tested after a further 11 weeks in drug free medium (week 28). In order to allow distinction of the previously treated K/EC32T cells and the newly treated cells in these experiments, newly drug exposed cells were named by the addition of the suffix T*. The effect on the previously treated K/EC32T cells of a

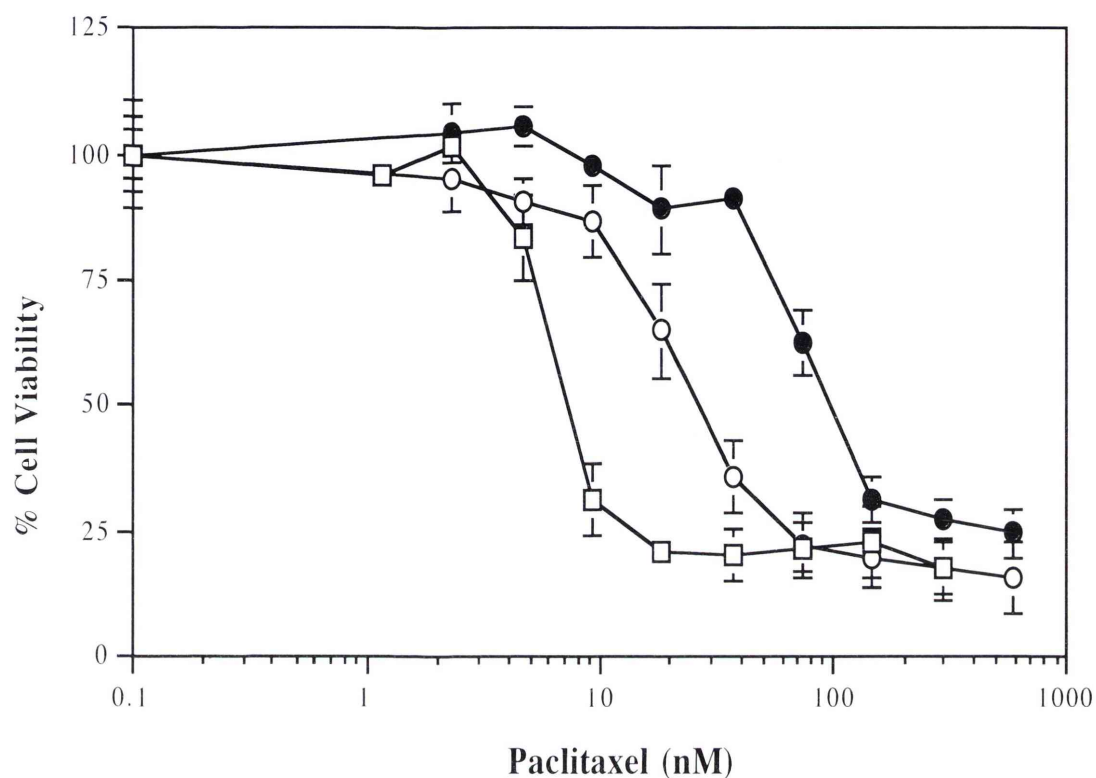


Figure 2.7 The effect of 3 day drug exposure on the K/EC32 cell line. K/EC32 cells were grown in drug free culture for 17 weeks then treated with 15ng/ml epirubicin for 3 days. Drug treated K/EC32T (●) and untreated K/EC32 cells (○) were then assayed for their resistance to paclitaxel and compared to the resistance of parental K562 cells (□). Points are the mean of triplicate wells and bars are the standard deviation.

second round of drug exposure at week 28, (designated K/EC32TT cells) was also examined. As seen previously drug treatment of the K/EC32 cell line again resulted in the induction of MDR (Figure 2.8). Thus the relative resistance to paclitaxel of the K/EC32T* cells was 5.6-fold compared to 2.3-fold in K/EC32 cells, that is an increase of 2.4-fold. The previously drug treated K/EC32T cells maintained a level of resistance equivalent to that of the freshly treated K/EC32T* cells. However the K/EC32TT cells which had undergone 2 drug treatments demonstrated a slightly further increase in relative resistance to 7.7-fold (Figure 2.8) (assays at week 28 were repeated three times with similar results).

A 3 day exposure of the K/EPR cells to 20ng/ml of epirubicin following 17 weeks in drug free culture also resulted in the induction of MDR. The resulting K/EPRT* cell line demonstrated 16.7 fold relative resistance to paclitaxel, which was a 4.2 fold increase compared to the untreated K/EPR cells (Figure 2.9). In contrast, 3 day exposure of the K/DNR cells to 20ng/ml of daunorubicin did not result in a large difference in relative resistance between the untreated K/DNR cells, (16.7-fold) compared to the 20 fold resistance in the treated K/DNRT* cells (Figure 2.10). These assays on K/EPR and K/DNR cells were repeated three times with similar results. These cells were at a high level of resistance prior to drug treatment and it may not be possible for the MDR levels to increase much higher.

The parental K562 cells were also treated with 5ng/ml of epirubicin, a concentration which gives a similar degree of cytotoxicity as does 15ng/ml to K/EC32 cell lines (data not shown). As shown in Figure 2.11, exposure of the parental K562 cells to drug resulted in no apparent difference in resistance between the K562 and K562T* cells (repeated three times with similar results). It is possible that an irreversible change occurs to produce the K562 MDR cell lines from the K562 cells and once this change having been effected, the MDR cell lines are also reversibly inducible.

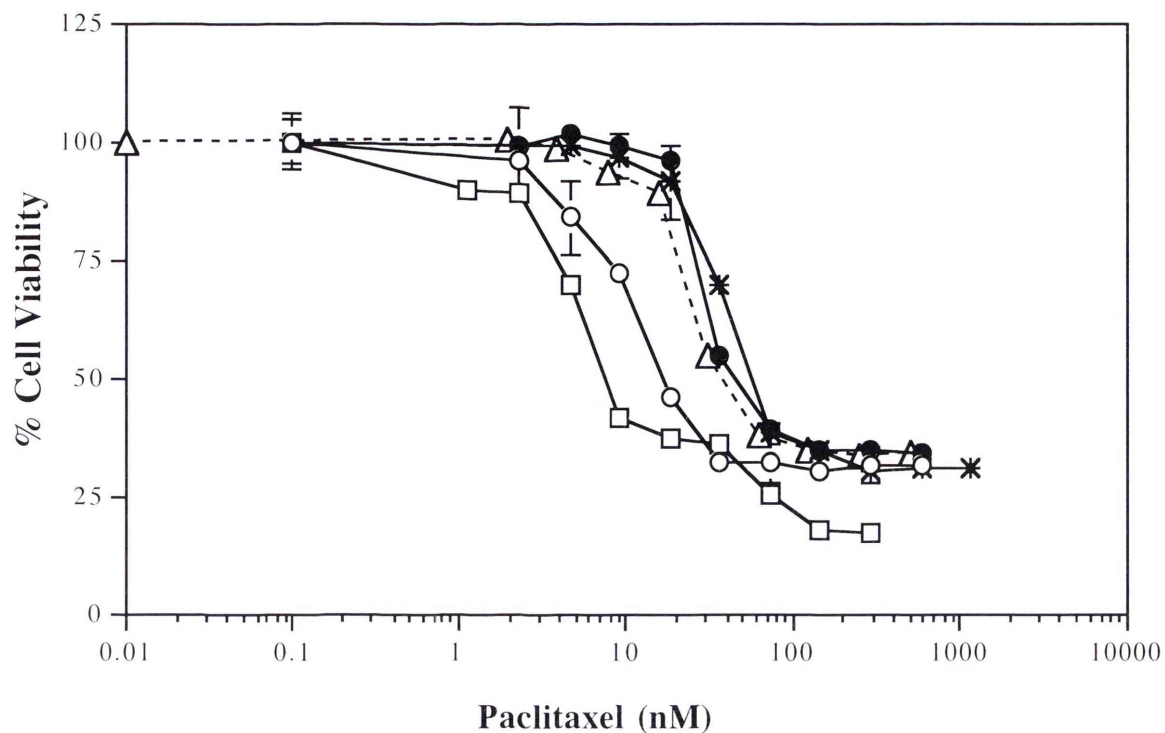


Figure 2.8 The effect of short-term drug exposure on the K/EC32 and K/EC32T cell lines. K/EC32 cells (○) were grown in drug free culture for 28 weeks before Paclitaxel cytotoxicity assay. K/EC32T cells (●) were grown in drug free culture for 17 weeks then treated with 15ng/ml epirubicin for 3 days, then assayed after a further 11 weeks in drug free culture. K/EC32T* (△) were grown in drug free media for 28 weeks then treated with 15ng/ml epirubicin for 3 days. K/EC32TT (*) cells were treated with 15ng/ml epirubicin for 3 days after 17 weeks in drug free culture and re-treated with 15ng/ml epirubicin for 3 days after 11 weeks in drug free culture. Paclitaxel cytotoxicity assays were performed on the cell lines and compared to the resistance of parental K562 cells (□) to examine the induction of drug resistance by drug treatment of the cells. Points are the mean of triplicate wells and bars are the standard deviation.

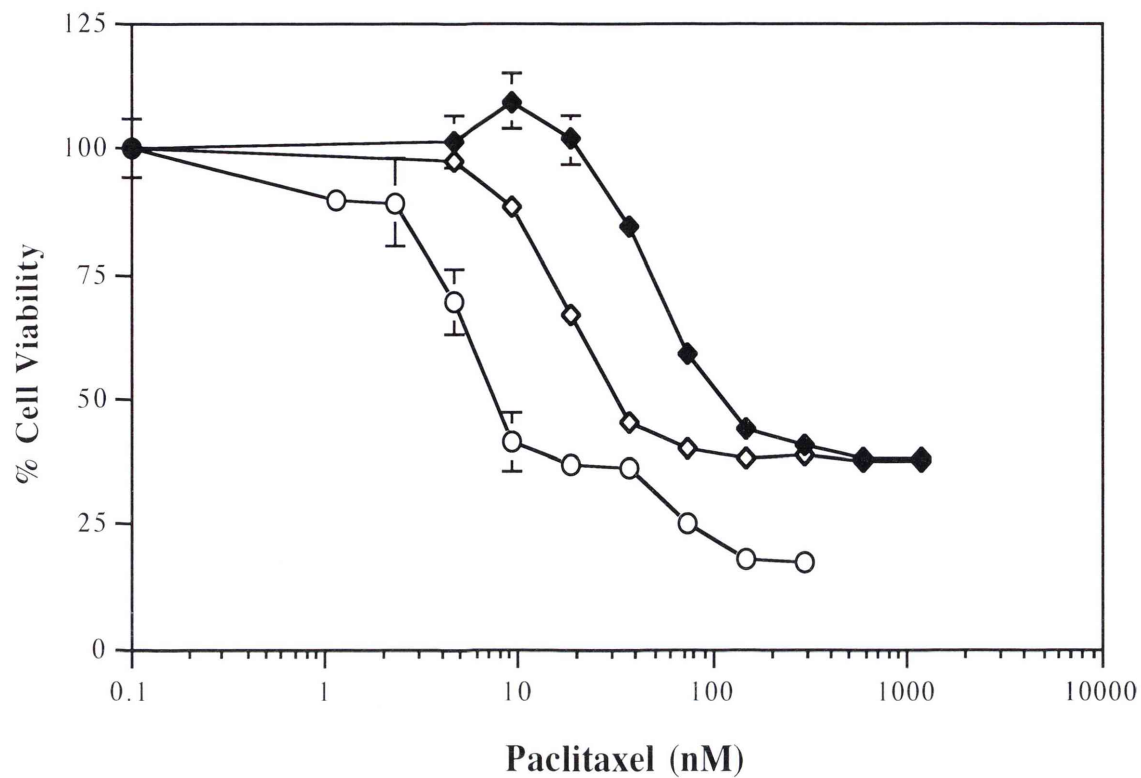


Figure 2.9 The effect of 3 day drug exposure on the K/EPR cell line. K/EPR cells were grown in drug free culture for 17 weeks then treated with 20ng/ml epirubicin for 3 days. Cytotoxicity assays to paclitaxel were performed for K/EPR (◇) and drug treated K/EPRT* (◆) and compared to the resistance of parental K562 cells (○) to examine the induction of drug resistance by drug treatment of the cells. Points are the mean of triplicate wells and bars are the standard deviation.

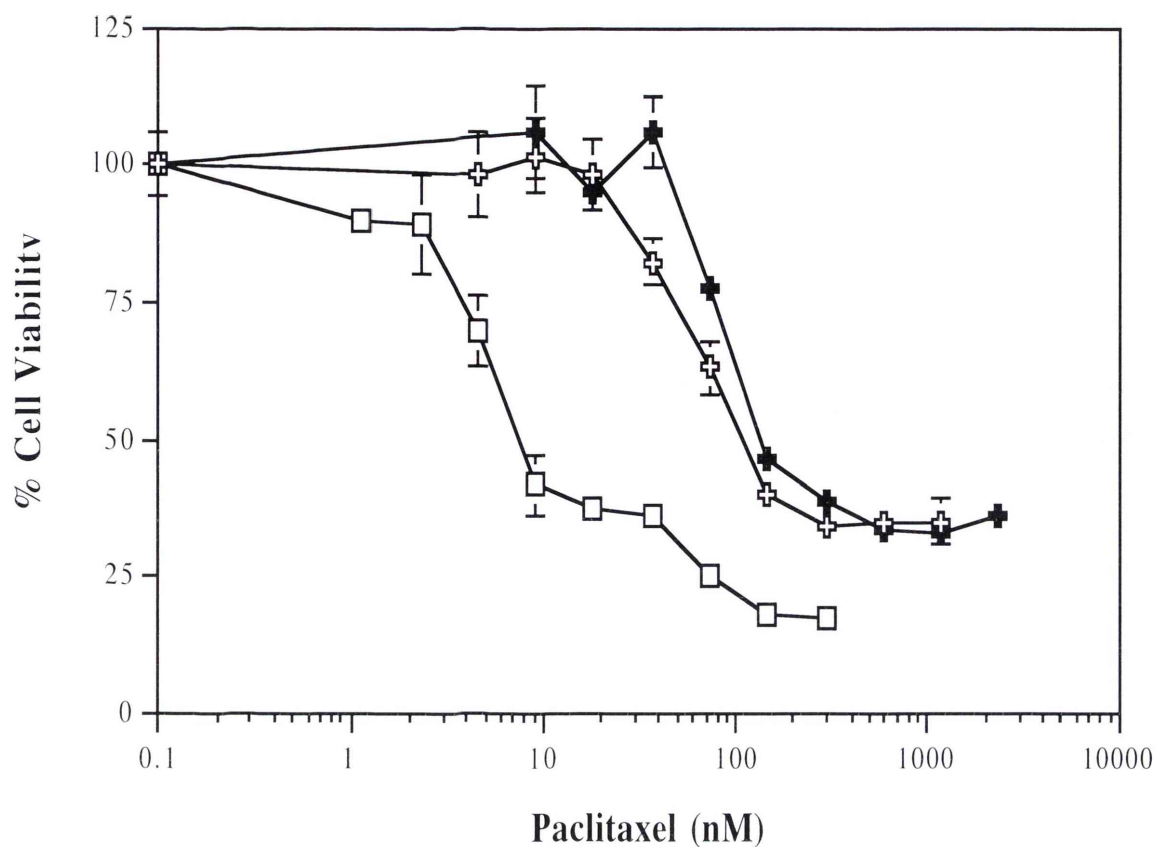


Figure 2.10 The effect of 3 day drug exposure on the K/DNR cell line. K/DNR cells were grown in drug free culture for 17 weeks then treated with 20ng/ml daunorubicin for 3 days. Cytotoxicity assays to paclitaxel were performed for K/DNR (†) and K/DNRT* (+) and compared to the resistance of parental K562 cells (□) to examine the induction of drug resistance by drug treatment of the cells. Points are the mean of triplicate wells and bars are the standard deviation.

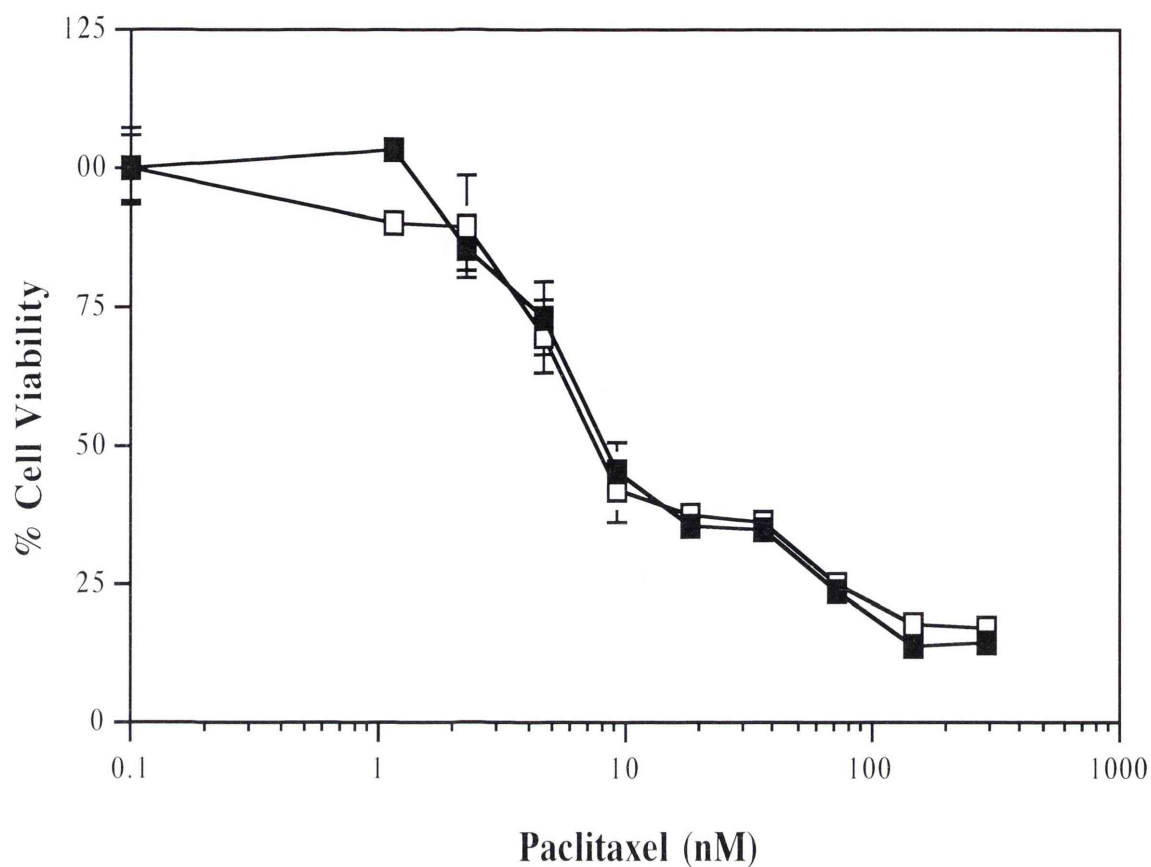


Figure 2.11 The effect of 3 day drug exposure on the K562 cell line. K562 cells grown in drug free culture treated with 5ng/ml epirubicin for 3 days. Cytotoxicity assays to paclitaxel were performed for K562 (□) and K562T* (■) cells to examine the induction of drug resistance by drug treatment of the cells. Points are the mean of triplicate wells and bars are the standard deviation.

2.3.6 *MDR1* mRNA levels in re-stimulated K562 MDR sublines

Northern Blots were performed on total RNA extracted from K562, non-drug treated MDR sublines and drug treated MDR sublines isolated at the same time the paclitaxel sensitivity was conducted, after 28 weeks for K/EC32, and 17 weeks for K/EPR and K/DNR (Figure 2.12). Table 2.3 shows the normalised levels of *MDR1* mRNA (relative to *GAPDH*) in the treated and untreated cell lines and also correlates these levels with the levels of paclitaxel relative resistance, determined at the time the RNA was extracted. Examination of this data shows a positive relationship between the level of *MDR1* mRNA present in the cells and the levels of MDR displayed by the cells at that time and confirm that the drug exposure that resulted in induced MDR produced a corresponding increase in *MDR1* mRNA levels in the cells. This result was reproduced in a subsequent experiment.

2.3.7 Induction by short term exposure to *MDR1* substrate drugs

To investigate if MDR could be induced by shorter exposure times, and by P-gp substrate drugs different to the original selecting drug, we treated the K562 MDR cell line K/EPR for either 4 or 16 hours to epirubicin or paclitaxel. The concentrations of epirubicin (the original selecting drug) or paclitaxel used were equivalent to an IC₂₅, as determined by treatment for 4 or 16 hours with serial dilutions of each drug then performing an MTT cell viability assay. Control treatments were set up in the same manner as the drug treatments except for the absence of drug.

Epirubicin, an anthracycline and an epimer of doxorubicin, is a P-gp substrate drug. Like other anthracyclines, epirubicin induces multiple intracellular effects that result in DNA damage in the cells (Nielsen *et al.*, 1996). This is achieved through intercalation of DNA base pairs resulting in inhibition of DNA replication and by direct inhibition of the enzymes DNA helicase, DNA polymerase and RNA polymerase (Wilson *et al.*, 1999).

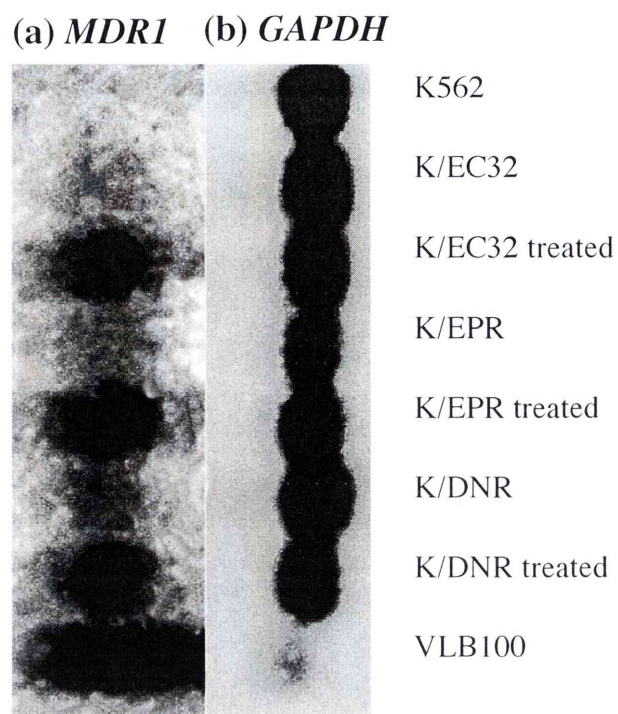


Figure 2.12 Northern blot for the determination of *MDR1* mRNA levels in K562 MDR cell lines. Total RNA from K562, and MDR sublimes before and after drug treatment was hybridised with (a) an *MDR1* DNA probe before stripping and re-hybridised with (b) a *GAPDH* RNA probe. VLB 100 RNA was used as a positive control.

Table 2.3 Correlation of *MDR1* mRNA and relative resistance levels in K562 MDR sublines following 3 day drug treatment after 28 weeks (K/EC32) or 17 weeks (K/DNR and K/EPR) in drug free culture.

| | <i>MDR1</i> mRNA | | Drug Resistance | |
|--------------|---|-------------------|---|-------------------|
| Cell Subline | Normalised <i>MDR1</i> Level ¹ | Relative Increase | Relative Paclitaxel Resistance [#] | Relative Increase |
| K/EC32 | 2.1 | 4.4 | 2.3 | 3.3 |
| K/EC32T* | 9.2 | | 7.7 | |
| K/EPR | 2.7 | 4.4 | 4.0 | 4.2 |
| K/EPRT* | 11.8 | | 16.7 | |
| K/DNR | 5.9 | 1.7 | 16.7 | 1.2 |
| K/DNRT* | 10.0 | | 20.0 | |

1. *MDR1* mRNA levels normalised to *GAPDH* mRNA levels.
2. Paclitaxel resistance levels shown are relative to that of untreated K562 cells and refer to the individual IC₅₀ drug concentrations.

Paclitaxel is also a P-gp substrate drug but its mode of action is quite different from that of epirubicin. It is a natural product drug obtained from the bark of *Taxus brevifolia*, a pacific yew tree (Niethammer *et al.*, 2001). Paclitaxel was the first taxane to be routinely used clinically (Luck *et al.*, 1997) and has applications including breast, lung and ovarian cancers (Niethammer *et al.*, 2001). Paclitaxel binds to microtubules causing formation of microtubulin bundles in interphase cells and spindle asters during mitosis. This results in increased polymer mass and stabilisation of microtubules leading to mitotic arrest and failure of chromosomes to segregate (Blagosklonny and Fojo, 1999, Reinecke *et al.*, 2000).

Short term exposure to epirubicin caused a temporary growth block and so the treated cells were allowed to recover for 3 days in drug free medium, before assaying MDR levels in a standard 4 day paclitaxel cytotoxicity assay. From these results it can be seen that treatment of K/EPR cells for as little as 4 or 16 hours with epirubicin (Figure 2.13) induced higher levels of MDR in these MDR cells (treatments performed four times with similar results). Cells pre-treated with paclitaxel, for 4 or 16 hours at concentrations giving similar inhibition to epirubicin also resulted in an increase in subsequent resistance to paclitaxel (Figure 2.14) ((treatments performed four times with similar results). In contrast to epirubicin, short term exposure to paclitaxel at these concentrations did not block growth after removal (data not shown), but paclitaxel exposed cells were also assayed following a 3 day drug free recovery period. The effect of 4 or 16 hours exposure to epirubicin or paclitaxel on subsequent sensitivity to paclitaxel is summarised in Table 2.4, from which it can be seen that both induction treatments resulted in a similar induction of MDR level.

2.3.8 Induction by Short Term Exposure to Cisplatin

To investigate if MDR could be induced by cytotoxic agents that are not P-gp substrate drugs, we treated the K/EPR cells for either 4 or 16 hours with cisplatin. The concentration of cisplatin used for treatments (500ng/ml) was equivalent to an IC₂₅, as determined by treatment for 4 or 16 hours with serial dilutions of cisplatin, then

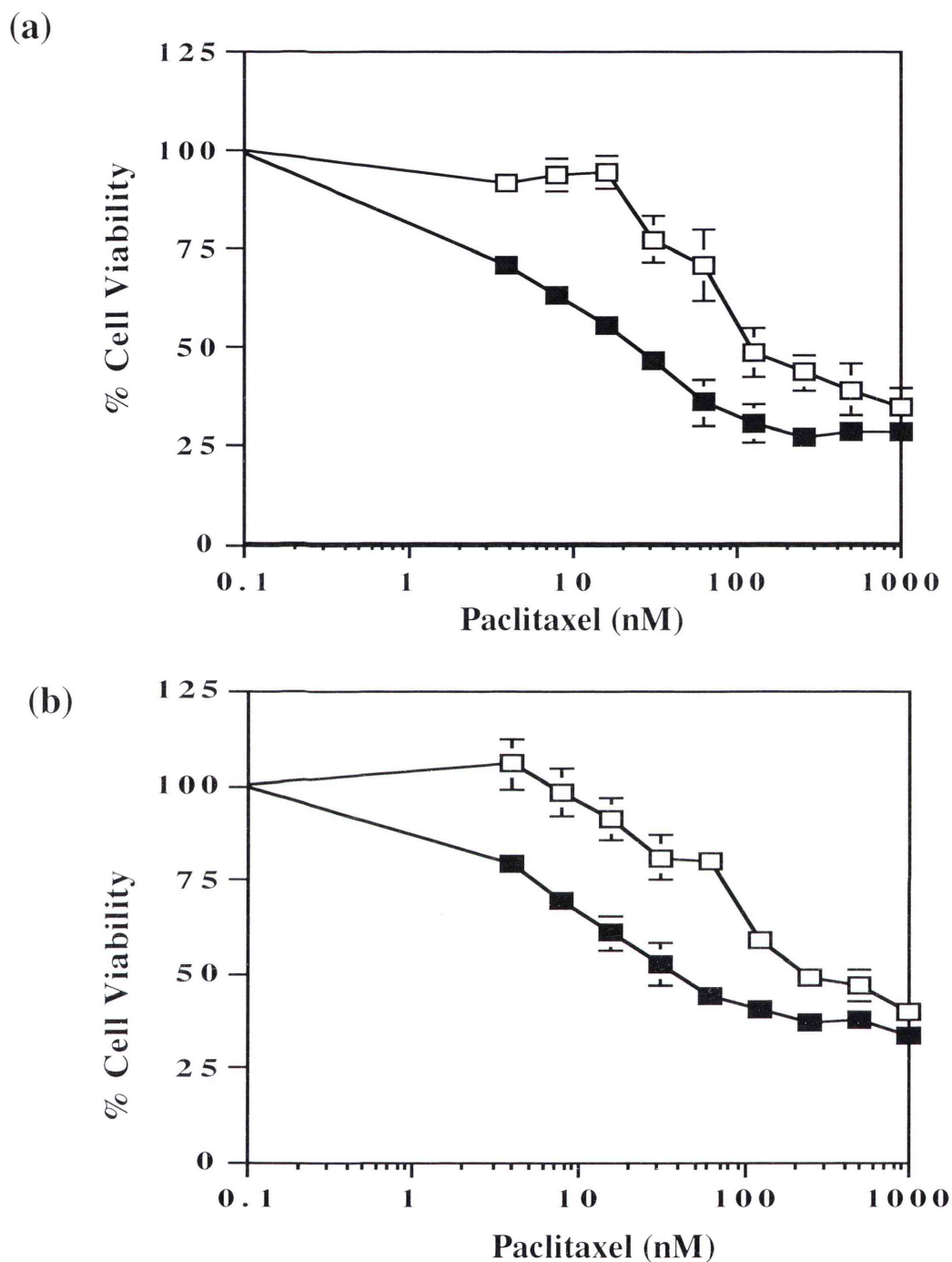


Figure 2.13 The effect of short-term epiubicin exposure on the K/EPR cell line. K/EPR were exposed to epiubicin for (a) 4 hours and (b) 16 hours then cytotoxicity to paclitaxel was assessed. Resistance of epiubicin treated cells (□) was compared to the resistance of control untreated K/EPR cells (■). Points are the mean of triplicate wells and bars are the standard deviation.

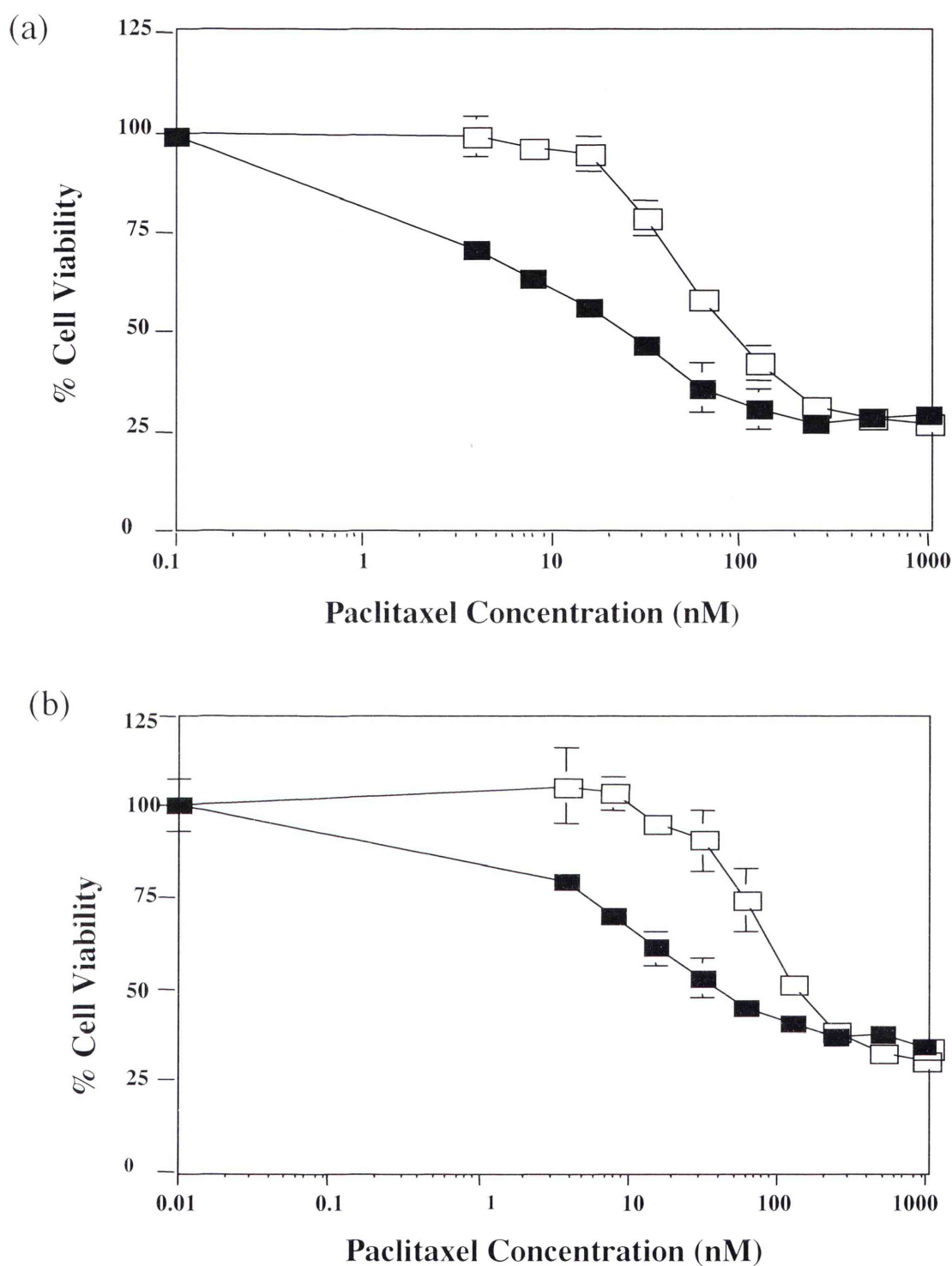


Figure 2.14 The effect of short-term paclitaxel exposure on the K/EPR cell line. K/EPR were exposed to paclitaxel for (a) 4 hours and (b) 16 hours then cytotoxicity to paclitaxel was assessed. Resistance of paclitaxel treated cells (□) was compared to the resistance of control untreated K/EPR cells (■). Points are the mean of triplicate wells and bars are the standard deviation.

Table 2.4 Comparison of K/EPR cell paclitaxel IC₅₀'s before and after 4 or 16 hour epirubicin or paclitaxel treatment.

| Drug treatment | Control IC₅₀ (nM) | Drug treated IC₅₀ (nM) | Relative Paclitaxel Resistance # |
|----------------------------|-------------------------------------|--|---|
| 4 hours epirubicin | 24 | 120 | 5.0 |
| 4 hours paclitaxel | 25 | 98 | 3.9 |
| 16 hours epirubicin | 40 | 220 | 5.5 |
| 16 hours paclitaxel | 40 | 140 | 3.5 |

resistance levels shown are relative to that of untreated K/EPR cells and refer to the individual IC₅₀ drug concentrations.

performing an MTT cell viability assay. Control treatments were set up in the same manner as the drug treatments except for the absence of drug.

Unlike epirubicin or paclitaxel, cisplatin is a non-P-gp substrate drug. The exact mechanism of cisplatin action is unknown, however it is thought that cisplatin causes DNA damage through the formation of a variety of covalent DNA adducts that result in DNA crosslinks (Ise *et al.*, 1999, Cohen, 2001). These cisplatin adducts are thought to deform the conformation of the DNA and thus inhibit or eradicate DNA replication and RNA transcription in the effected cell (Brabec and Kasparkova 2002). The reason for inclusion of cisplatin in the current induction studies even though, as previously stated, it is not a P-gp substrate drug, was because previous studies by other groups have indicated that cisplatin has induced P-gp levels both *in vitro* (Ohga *et al.*, 1998, Chaudhary and Roninson, 1993) and *in vivo* (Demeule *et al.*, 1999).

Like paclitaxel, short term exposure to cisplatin at similar cytotoxic concentrations did not block growth after removal, but cells exposed to these drugs were also assayed following a 3 day drug free recovery period. Results for cisplatin (Figure 2.15) indicate no induction of MDR has occurred (treatments performed four times with similar results). As was done previously with paclitaxel, for cells pre-treated with cisplatin, assay of MDR level in a standard 4 day paclitaxel cytotoxicity assay performed immediately after the 4 or 16 hour drug exposures gave the same result (data not shown).

Recent reports have indicated that in some cell lines pre-exposure to cisplatin can sensitise the cells to paclitaxel (Yamamoto *et al.*, 2000). In this study paclitaxel is being used to assess resistance levels after cisplatin treatment, thus an increase in resistance after cisplatin treatment may be masked. To overcome this, the resistance levels of cisplatin exposed cells were also assessed with vinblastine, however these results also indicated that no induction of drug resistance had occurred (Figure 2.16) (this result was reproduced in a subsequent experiment). Thus both of the P-gp substrates epirubicin and paclitaxel induced MDR levels, but the non-P-gp substrate cisplatin at similar inhibitory concentration had no inducing effect. Moreover treatment of the K/EPR cells with

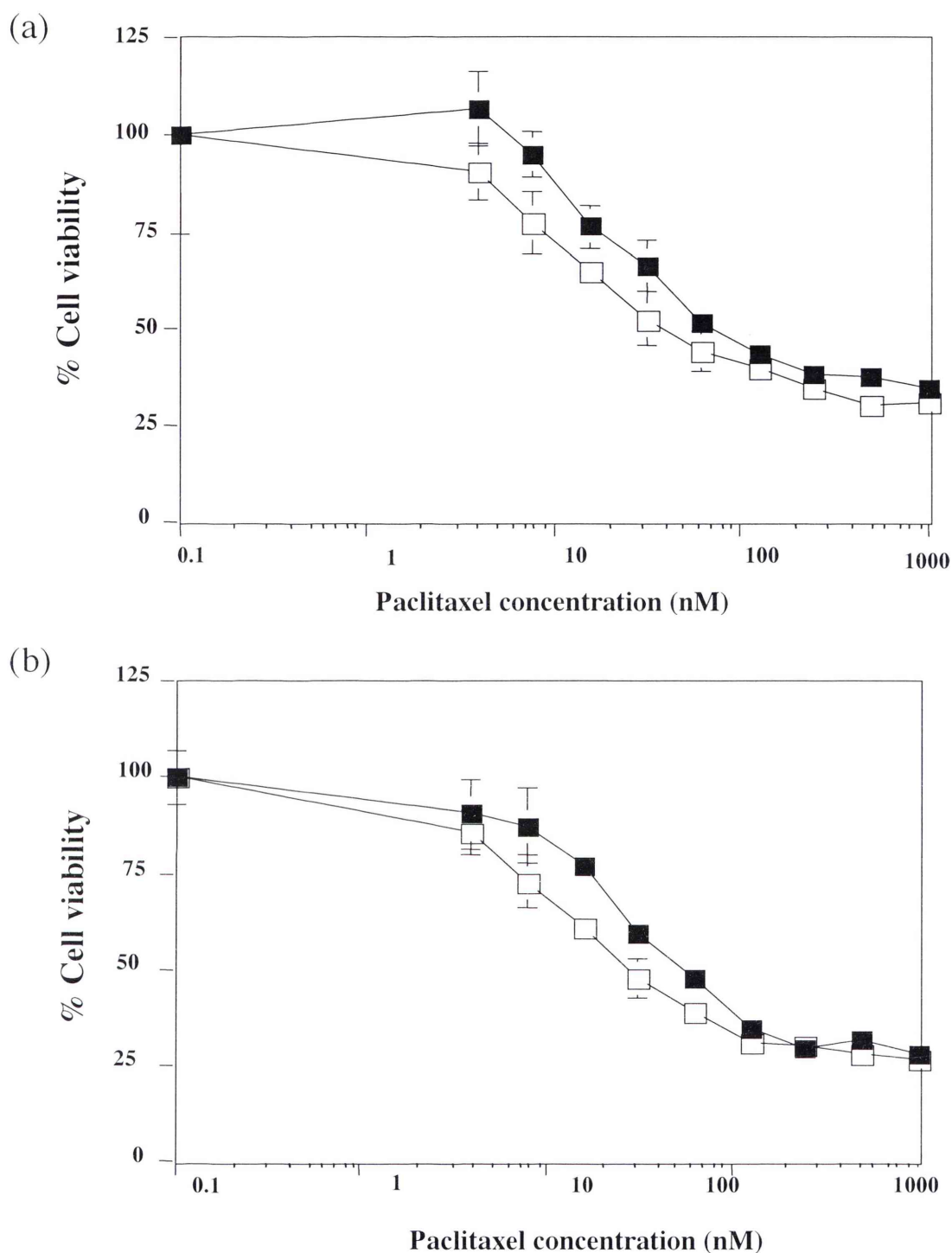
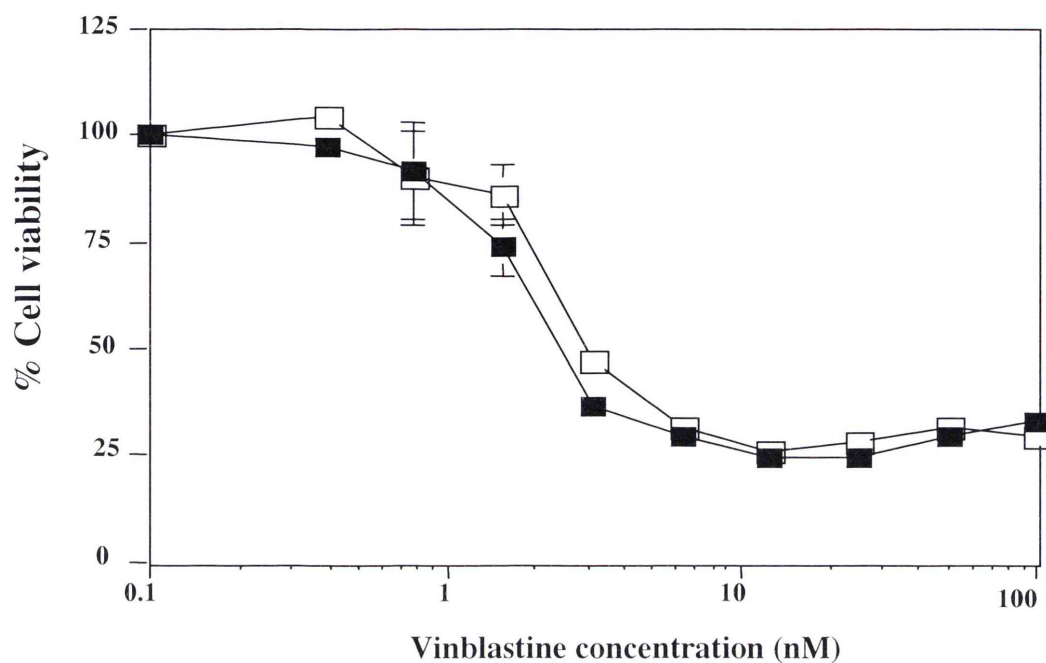


Figure 2.15 The effect of short-term cisplatin exposure on the K/EPR cell line. K/EPR were exposed to cisplatin for (a) 4 hours and (b) 16 hours then cytotoxicity to paclitaxel assessed. Resistance of drug treated cells (□) was compared to the resistance of control untreated K/EPR cells (■). Points are the mean of triplicate wells and bars are the standard deviation.

(a)



(b)

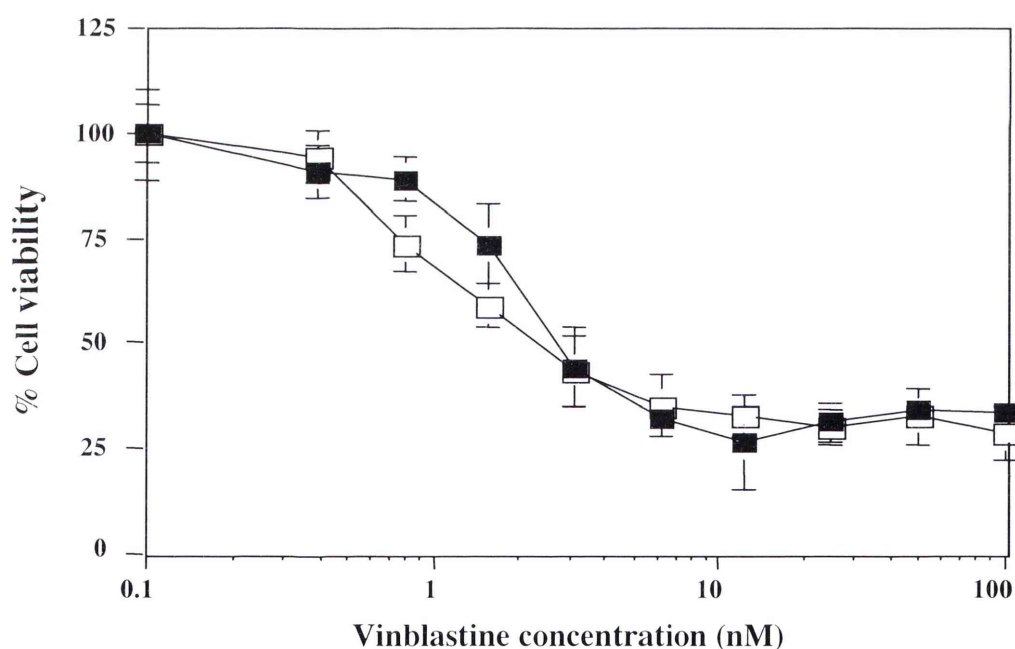


Figure 2.16 The effect of short-term cisplatin exposure on the K/EPR cell line. K/EPR were exposed to cisplatin for (a) 4 hours and (b) 16 hours then cytotoxicity to vinblastine assessed. Resistance of drug treated cells (□) was compared to the resistance of control untreated K/EPR cells (■). Points are the mean of triplicate wells and bars are the standard deviation.

cisplatin prior to paclitaxel exposure in the cytotoxicity assay resulted in a slight decrease in the paclitaxel resistance and this is consistent with the previous observations of Yammamoto *et al.*, (2000).

2.3.9 Induction by short term exposure to rifampacin

Rifampacin, also known as rifampin, is a semi synthetic antibiotic derived from rifamycin B which is produced by the fermentation of a strain of *Streptomyces mediterranei* (reviewed in Sensi, 1983). Rifampacin acts by binding to the B-sub-unit of bacterial DNA-dependent RNA polymerase thus preventing the initiation of DNA transcription (Fahr *et al.*, 1985). Previous studies have indicated that rifampacin treatment can induce intestinal P-gp (Greiner *et al.*, 1999, Westphal *et al.*, 2000, Geick *et al.*, 2001).

As rifampacin was found not to be cytotoxic to the K/EPR cell line, three concentrations of rifampacin were chosen to both cover and exceed the concentrations used in the literature. Chosen rifampacin concentrations were 10 μ m, 30 μ m and 100 μ m. Control treatments were set up in the same manner as the drug treatments except for the absence of drug. However as shown in Figure 2.17, as for cisplatin, short term exposure of the K/EPR cells to rifampacin at a variety of concentrations also did not induce MDR levels (this result was reproduced in a subsequent experiment).

2.3.10 Effect of short term exposure to the histone deacetylase inhibitor TSA

A previous study by Jin and Scotto, (1998) demonstrated that the *MDR1* promoter could be induced in SW620 colon carcinoma cells by exposure to the histone deacetylase inhibitor TSA. Other published studies using TSA to activate genes use concentrations

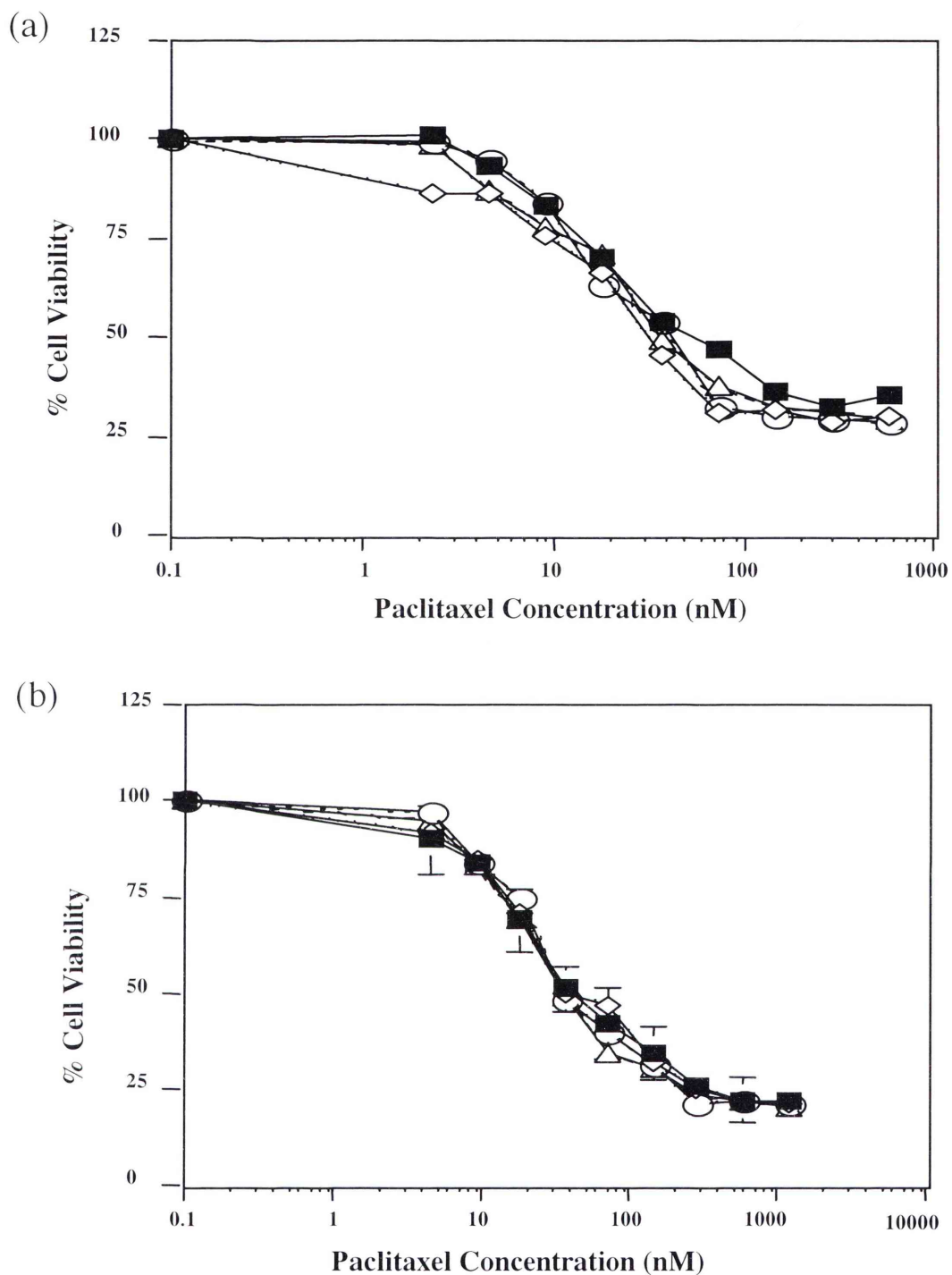


Figure 2.17 The effect of short-term rifampacin exposure on the K/EPR cell line. K/EPR cells were exposed to rifampacin for (a) 4 hours and (b) 16 hours at $10\mu\text{M}$ (◇), $30\mu\text{M}$ (○), $100\mu\text{M}$ (Δ), then cytotoxicity to paclitaxel was assessed and resistance compared to control untreated cells (■) to examine the induction of drug resistance by the drug treatment of the cells. Points are the mean of triplicate wells and bars are the standard deviation.

of TSA from 3nM to 500nM for 16-24 hours (Jin and Scotto, 1998; Jones *et al.*, 1998; Cameron *et al.*, 1999). Therefore K562 and K/EPR cells were incubated for 16 hours in concentrations of TSA varying from 0 to 1000nM. To determine the effects TSA had on the growth of the cells, TSA treated cells were counted after a three day recovery period in normal growth medium. To determine the effect TSA exposure has on K562 and K/EPR MDR levels, the sensitivity of the TSA treated cells to epirubicin was assessed.

As shown in Figure 2.18a TSA slightly inhibits the growth of the K562 cells, with viability decreasing from 100% to 85% at 50nM TSA, then remaining approximately constant to 500nM TSA. From Figure 2.18b it can be seen that the IC₅₀s obtained by treatment with epirubicin of untreated K562, ethanol control, 500nM and 1000nM TSA treated cells were all approximately 11nM, whereas the cells treated with 50nM or 200nM TSA had a 2.2 fold higher IC₅₀ of 24nM epirubicin. These experiments were repeated 3 times with similar results. The results indicate an induction of MDR levels by pre-treatment with low inhibitory TSA concentrations, which is extinguished at high concentrations.

Figures 2.19a and 2.19b show the corresponding results for K/EPR cells. TSA has less inhibitory effect on the growth of K/EPR cells (Figure 2.19a) than on the K562 cells, requiring 500nM to 1000nM to reduce growth by 10 to 15%. As shown in Figure 2.19b, TSA concentrations up to 500nM had no effect on MDR levels (IC₅₀ of 35nM epirubicin). Treatment with 1000nM TSA slightly decreased the IC₅₀ to 18nM. This experiment has been performed 3 times with similar results. Thus these results suggest that while TSA does not appear to upregulate MDR levels in the K/EPR cell line, it may increase drug resistance levels slightly in the more drug sensitive K562 cells.

2.4 SUMMARY OF RESULTS

The relative resistance levels of the cell lines to be used was confirmed. Initial testing of the cytotoxicity levels of the K562 MDR cell lines showed that K/EC32 cells displayed very low level drug resistance, the K/EPR cells demonstrated medium drug resistance and K/DNR cells had a higher drug resistance. These cell lines demonstrated resistance to the specific P-gp substrate paclitaxel, which was reversible by verapamil, indicating

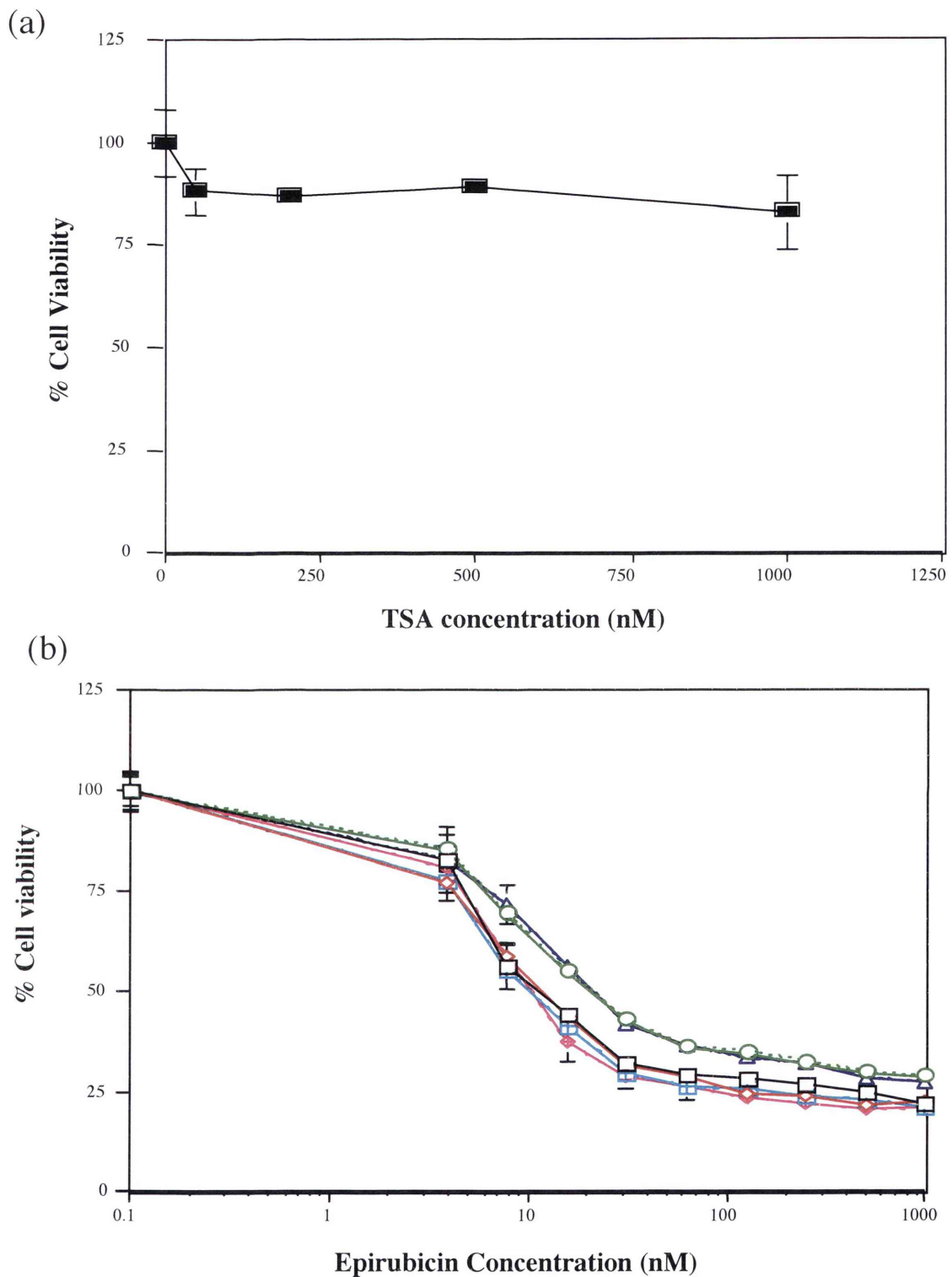


Figure 2.18 Effect of TSA on growth and epirubicin resistance of K562 cells. Exponentially growing K562 cells were treated with 0, 50, 200, 500 and 1000nM TSA for 16 hours and assayed for effect on (a) cell growth (■) and (b) resistance to epirubicin following treatment with TSA (○ = 50nM, △ = 200, ▢ = 500, ◇ = 1000nM) or no TSA (◇ = ethanol control, = media control).

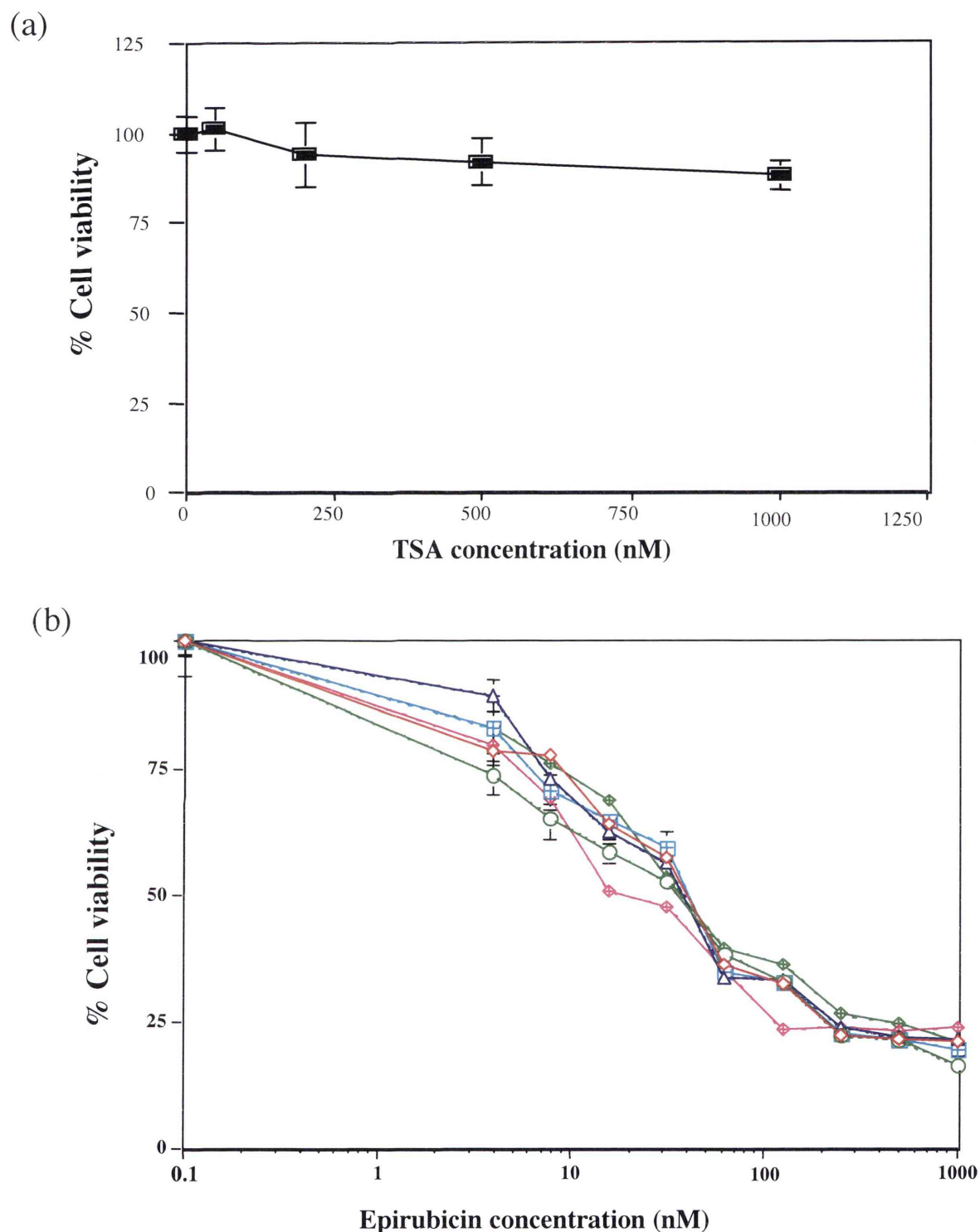


Figure 2.19 Effect of TSA on growth and epirubicin resistance of K/EPR cells. Exponentially growing K/EPR cells were treated with 0, 50, 200, 500 and 1000nM TSA for 16 hours and assayed for effect on (a) cell growth (■) and (b) resistance to epirubicin following treatment with TSA (O = 50nM, Δ = 200, ◻ = 500, ◈ = 1000nM) or no TSA (◊ = ethanol control, ◀ = media control).

that MDR in these lines is P-gp mediated. The low MDR levels exhibited by these cell lines is consistent with the relatively low drug concentrations used for their development.

The levels of drug resistance displayed by the K562 MDR sublines correlated with the level of the *MDR1* mRNA in the cells. Increased amounts of *MDR1* mRNA in some cell lines can occur as a result of amplification of the *MDR1* gene copy number in the cells (Kohno *et al.*, 1994). Gene amplification frequently occurs *in vitro* during selection of MDR cell lines, especially when the cell are exposed to high, clinically irrelevant concentrations of drugs (Morrow *et al.*, 1994; Kohno *et al.*, 1994; Shen *et al.*, 1986). However gene amplification is usually avoided when low, clinically relevant concentrations of drug are used to isolate MDR cells (Shen *et al.*, 1986) as was the case for the cell lines used for this study (Marks *et al.*, 1993; Hargrave *et al.*, 1995). Consequently, Southern hybridisations using an *MDR1* DNA probe performed on slot blots of the genomic DNA from the cells showed that the K562 MDR cell lines had an *MDR1* gene copy number equivalent to the parental K562 cells, indicating that amplification of the *MDR1* gene had not occurred in the K562 MDR cell lines.

Periodic assessment of resistance levels in the cell lines through cytotoxicity assays revealed that drug resistance in the MDR cell lines declined over time in drug free culture. However resistance levels could be restimulated in each of the MDR cell lines by a single short-term treatment with the original selecting drug. Furthermore upregulation of resistance in the MDR sublines induced by short-term drug exposure was accompanied by increased expression of *MDR1* mRNA. In contrast to the induction of drug resistance in the K562 MDR sublines, treatment of the parental K562 cells with a dose of epirubicin that has a level of cytotoxicity equivalent to the concentrations used to treat the MDR cell lines, was unable to induce MDR, indicating a fundamental difference between the K562 cells and its derived MDR sublines.

In our experiments we found that only P-gp substrates were able to induce *MDR1* expression, with no induction seen with the non-Pgp drug cisplatin. As studies have shown that exposure of cells to cisplatin can sensitise cells to paclitaxel treatment (Yamamoto *et al.*, 2000) it is possible that the apparent failure of MDR induction by cisplatin in the K/EPR cells could be due to increased paclitaxel sensitivity after

cisplatin treatment. To overcome the possibility of an induction of MDR being masked in cisplatin treated cells by using paclitaxel to assay any changes in cell resistance, vinblastine cytotoxicity assays performed on cisplatin treated cells, however this also indicated no induction of drug resistance. Induction of resistance was also not seen when the K/EPR cells were exposed to rifampacin, a drug which binds to the SXR receptor which has been shown to bind to a response element approx 8kb upstream of the *MDR1* gene in the human colon carcinoma cell line LS174T cell line (Geick *et al.*, 2001).

Since different MDR levels in the parental K562 and drug resistant sublines may reflect differences in their levels of histone acetylation and chromatin structure, the effect of TSA, a specific inhibitor of histone deacetylase (Yoshidai *et al.*, 2001) on MDR levels in K562 and K/EPR cells was investigated. The results indicated that exposure of K562 cells to 50nM to 200nM TSA, increased the level of epirubicin resistance approx 2.2 fold. However no corresponding increase was seen in the K/EPR cell line. This result may indicate that in the drug sensitive K562 cells, TSA inhibition of histone deacetyltransferase led to increased acetylation and subsequent activation of *MDR1* in these cells. However the absence of any similar effect in the K/EPR MDR cell line indicates a difference in chromatin structure in the two cell lines.

CHAPTER 3

Investigation of Binding to Transcription Factor Sites in the *MDR1* Promoter

3.1 INTRODUCTION

The ability of the K/EPR cells to induce MDR levels after as little as 4 hours of drug treatment suggested that a transcriptional mechanism of induction may be involved. As the Y-box has previously been implicated in *MDR1* up-regulation (Cornwell *et al.*, 1993), the *in vitro* levels of nuclear proteins that bind to the Y-box in K562 cells and MDR sublines before and after drug treatment were examined using Electrophoretic mobility shift assays (EMSAs). The identity of the Y-box binding protein has been the subject of contention in much of the literature, and appears to differ in different cell lines. To determine the identity of the transcription factor binding to the Y-box of the *MDR1* promoter of these cells, single stranded YB-1 binding sequences and anti-NF-Y antibodies were used in EMSAs. Single stranded YB-1 binding sequences were also included as it has been shown that YB-1 binds preferentially to single stranded DNA (Tafuri and Wolffe, 1992).

The -55GC box appears to be essential in terms of basal *MDR1* transcription and contains a consensus binding sequence for the transcription factor Sp1. Previous work has demonstrated the importance of this region through co-transfection of *Drosophila* cell lines, which do not constitutively express Sp1, with an *MDR1*-luciferase reporter and Sp1 construct (Cornwell and Smith, 1993). The cells transfected with both the

MDR1 reporter and an Sp1 construct showed 15 fold higher reporter expression than cells transfected with the *MDR1* reporter alone. Therefore levels of Sp1 binding to the –55GC box in K562 cells and MDR sublines were examined by EMSAs. Relative binding levels were also compared before and after drug induction of the K/EPR cell line.

To test transcriptional transactivation in a more *in vivo* model, K562 and K/EPR cells were also transfected with a *Luc* reporter vector containing an *MDR1* promoter (–88/+76) relative to the transcription start site (TSS), encompassing both the –55GC box and the Y-box, cloned upstream of the *Luc* gene. The reporter activity was then compared with and without drug treatment. Transient transfections were also carried out using *MDR1* constructs that also contain either +283/+392 or +329/+455 regions (relative to the TSS) of the *MDR1* gene cloned into a cloning site downstream of the *Luc* gene since previous preliminary studies have indicated a possible negative regulatory element within this region of the *MDR1* gene (Byrne, 2001; Cassano, Honours 2000).

A region at approx –120 to –112, containing a CAAT box (–118 to –113) has been reported to act as a negative regulatory element (NRE) based on the demonstration in MDR K562/ADM cells of an increase in promoter reporter activity upon mutation of this region (Ogura *et al.*, 1992). Transfection of drug sensitive MCF-7 cells with a –118 CAAT box deleted promoter reporter construct also resulted in a 12 fold increase in *MDR1* promoter activity compared to wild type promoter, indicating repression of *MDR1* transcription in MCF7 cells by a CAAT binding factor to this region (Ogretmen and Safa, 1999). Transfection of MDR MCF-7/Adr cells with the CAAT deleted promoter had little effect compared to wild type, indicating that the repressor was not active in the MDR cell line. To determine the relative levels of a possible repressor protein binding to the NRE at approx –120 to –112 of the *MDR1*, EMSAs were carried out on nuclear extracts from K562 and the multidrug resistant K/EPR cells using a binding probe encompassing this region.

3.2 MATERIALS AND METHODS

3.2.1 EMSAs

EMSAs are a method of identifying possible DNA-protein interactions. They involve labelling DNA of a transcription factor binding sequence and incubating this with extracted nuclear proteins. These reactions are then run on a non-denaturing polyacrylamide gel and any DNA-protein complexes formed will be retarded in the gel whilst free DNA will migrate at a much faster pace. Appropriate protein specific antibodies can also be pre-incubated with the nuclear protein extract to confirm the identity of the DNA-protein complexes formed. Specific antibodies will bind to the protein, thus increasing the size of DNA-protein complexes and this will result in a shift in the complex on the gel.

3.2.1.1 Nuclear Extract Preparation

The nuclear extraction method was adapted from Andrews and Faller (1991). All steps were carried out at 4°C. 1×10^7 exponentially growing cells were washed in cold PBS then cell pellets were resuspended evenly in 400 μ l of cold buffer containing 10mM HEPES pH7.9 at 4°C, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.2mM PMSF (Sigma Aldrich, Australia), 0.75mM spermidine (Sigma Aldrich, Australia), 0.15mM spermine (Sigma Aldrich, Australia), 1mM benzamidine (Sigma Aldrich, Australia), 10 μ g/ml aprotinin (Sigma Aldrich, Australia) and 10 μ g/ml leupeptin (Sigma Aldrich, Australia). The cells were allowed to swell on ice for 10 min, after which 25 μ l of 1% NP-40 was added drop wise with mixing to lyse cells. The cell lysate was vigorously vortexed for 10 seconds and spun for 10 seconds at top speed in a bench top microfuge. The nuclear pellet was resuspended in 50 μ l of buffer containing 20mM HEPES pH7.9 at 4°C, 25% glycerol, 1.2mM MgCl₂, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, 2mM benzamidine, 5 μ g/ml aprotinin and 5 μ g/ml leupeptin. The volume of the cell lysate was determined and an equal volume of the same buffer containing 840nM KCl was added (to ensure a final concentration of 420nM). The samples were rocked for 1 hour to allow high salt extraction of the nuclear proteins, then the extracts

were centrifuged at 15, 000g for 5 min and the supernatant containing the nuclear proteins was collected. The nuclear extract was dialysed in dialysis tubing for 1.5 hours against 3 changes of a 100 fold excess of dialysis buffer containing 20mM HEPES pH7.9 at 4°C, 20% glycerol, 100mM KCl, 0.2mM EDTA and 0.2mM EGTA. In later experiments the dialysis step was omitted since trials showed no difference between the binding of dialysed and undialysed extracts. Protein concentrations of the dialysed nuclear extracts were determined by the method of Bradford (1976).

3.2.1.2 Preparation of Labelled Binding DNA

Equimolar amounts of appropriate forward and reverse partially complimentary oligonucleotides were annealed in 1x one-phorall buffer (Pharmacia, Sweden) by incubation at 90°C for 5 min then allowing to cool slowly. Completion of synthesis and ³²P-labelling was performed in 10µl reactions containing 1pmol of annealed oligonucleotides, 1µl of one-phorall buffer, 2µl of dCTP label mix (1mM dGTP, 1mM dATP, 1mM dTTP, 50mM Tris pH 7.5, 250mM NaCl, 25mM β-mercaptoethanol) 2.5µl of α-32P-dCTP (10mCi/ml, 3000µCi/mmol) and 0.5µL of Klenow DNA polymerase (5U/µl). After incubation at room temperature for 20 min, 1µl of cold chase (1mM dCTP, 1mM dGTP, 1mM dATP, 1mM dTTP, 50mM Tris pH 7.5, 250mM NaCl, 25mM β-mercaptoethanol) was added and incubated at room temperature for 5 min, to ensure the oligonucleotides were completely end filled.

The labelled double stranded DNA fragments were separated from unincorporated nucleotides by electrophoresis at 100V on 12% polyacrylamide gels (20 acrylamide: 1 bisacrylamide) with 1x TBE running buffer. Labelled DNA fragments were localised by autoradiography, sliced from the gel and eluted from the gel slices overnight in 400µl of TE at 4°C. The fragments were then ethanol precipitated by the addition of 0.1 volumes of 3M Sodium acetate pH6.0 and 2.5 volumes of 100% ethanol and incubated at -70°C for 20 min. The tubes were then centrifuged at 12, 000xg and the pelleted labelled DNA washed with 100µl of 70% ethanol and allowed to air dry before being resuspended in 200µl of sterile water.

Single stranded YB-1 oligonucleotide was ^{32}P -end labelled in 20 μl reactions containing 10pmoles single stranded YB-1 oligonucleotide, 1x T4 polynucleotide kinase buffer, 5 μl of $\gamma^{32}\text{P}$ -dATP (10mCi/ml, 3000 $\mu\text{Ci}/\text{mmol}$) and 10U T4 Polynucleotide Kinase. This was incubated at 37°C for 45min followed by 68°C for 10min. An equal volume of 100% formamide was added to the label reaction, which was then vortexed and heated to 55°C to disrupt secondary structure. The reaction was then electrophoresed on a 19% denaturing polyacrylamide gel at 780V at 55°C (as described in Sambrook *et al.*, 1988), and the labelled oligonucleotide localised by autoradiography and excised from the gel. Ten volumes of H_2O were added to gel fragments and rocked at room temperature for 10min to remove urea. This was replaced with 10 volumes of fresh H_2O and rocked overnight at room temperature.

Unlabelled competitor DNA molecules were prepared by annealing and end filling the forward and reverse oligonucleotides as described for the labelled fragments, except that cold chase was used in place of dCTP label mix and no $\alpha^{32}\text{P}$ -dCTP was used. The double stranded competitor DNA was ethanol precipitated as described above. The oligonucleotides used to prepare the binding sequences are shown below.

| | |
|----------------------|--|
| Y-box | 5' -TG TGG TGAGGCTGATTGG CGACTAACCGACCCGTCCTTG-5' |
| Single stranded YB-1 | 5' -GTGATTGGCCAAAG |
| -55GC box | 5' -ACAGCGCCGGGGCGTGGGCTGAGCACA GACTCGTGT-5' |
| -120/-113 NRE | 5' -ATCAGCATTTCAGTCAAT GTAAGTCAGTTAGGCCCG- 5' |
| USF | 5' -GATCCGAATTCCACGTG TAAGGTGCACTGCCTCTTAAG-5' |

3.2.1.3 Binding Reactions

EMSAs were performed in 20 μ l reactions containing 15fmol of labelled DNA, 50 μ g BSA, 1 μ g poly(dI-dC).poly(dI-dC) (Sigma Aldrich, Australia) and 3-5 μ g of nuclear extract in a buffer containing 8mM HEPES (pH 7.9), 60mM KCl, 8% glycerol, 0.3mM EDTA, 0.3mM EGTA, 5mM MgCl₂, 0.05% NP-40 and 0.5mM DTT. The reactions were incubated at 20°C for 30 min. For competitor reactions, 100-fold excess of competitor DNA was added to the reactions prior to incubation. For supershift reactions 1 μ g anti-NF-YA antibody (Rocklands, USA) was preincubated with nuclear extracts for 3 hours at 4°C prior to adding the label.

The reactions were then electrophoresed at 4°C on a pre-run 6% polyacrylamide gel (80 acrylamide: 1 bisacrylamide) at 300V with 0.25x TBE running buffer that was recirculated every 30 min. The gels were fixed in 10% glacial acetic acid for 10 min, washed in water briefly, then transferred to Whatman 3MM paper and vacuum dried. The dried gel was autoradiographed by exposure to X-ray film (Kodak, Australia) with 2 intensifying screens (Dupont Quanta III) or to a Biorad phosphorImager screen (BI). The resulting images were analysed using the Molecular Analyst program (BioRad). Levels of binding complexes were estimated by determining the amount of specific complexes formed as a percentage of the total radioactivity loaded onto the gel. To compensate for differences in yields of factors in different nuclear extracts, the amounts of complexes of interest were normalised by dividing by the amount of complex formed with the USF binding probe by the same extract.

i.e.

$$\frac{\frac{\text{Total bound specific X complexes}}{\text{Total DNA (bound+unbound)}}}{\frac{\text{Total bound USF complexes}}{\text{Total DNA (bound+unbound)}}}$$

3.2.2 Transient Transfections

K/EPR cells were treated with and without 160ng/ml epirubicin for 16 hrs, then washed in serum free RPMI-1640 medium and transfected with MDR-*Luc* constructs. The *Luc*

construct contains an *MDR1* promoter comprising the sequence between -88 and +76 of the *MDR1* gene, cloned upstream of the *luc* gene in the pGL3 basic vector (Promega). The *Luc3AB* and *Luc3BC* constructs contain the *Luc1 MDR1* promoter plus *MDR1* gene sequences +283/+392 and +329/+455 bp downstream of the transcription start site respectively, cloned downstream of the *luc* gene. Cells were co-transfected with a CMV- β -galactosidase control reporter vector (generously provided by Dr Merlin Crossley, University of Sydney) for normalization purposes. Transfections were carried out in 24 well plates in triplicate. Serum Free RPMI-1640 (98 μ l) and DMRIE-C (Life Technologies) (2 μ l) was added to each well and mixed with 100 μ l media containing 0.4 μ g of *Luc1*, *Luc3AB*, or *Luc3BC* constructs and 0.5 μ g of CMV- β -galactosidase reporter vector DNA. Control (empty vector) transfections containing 0.4 μ g of pGL3-Basic vector (Promega) were also carried out. The reaction mixes were incubated at room temperature for 45 minutes. Cells (2.4×10^4) suspended in 40 μ l of serum free media were then added each well and incubated for 4-5 hours at 37°C, 5% CO₂, after which 400 μ l of RPMI-1640/15% FCS was added to each well and plates incubated for a further 48 hours.

3.2.2.1 Reporter Assays

Transfected cells were removed to microcentrifuge tubes and collected by centrifugation at 500g for 5 minutes to remove the media, then washed in 150 μ l PBS and re-centrifuged as above. Cells were then lysed by addition of 150 μ l of 1X Reporter Lysis Buffer (Promega) and frozen at -80°C until ready to be assayed. When ready to assay, tubes were thawed and centrifuged at 9000g for 5 minutes, to clear the supernatant to be assayed.

Galactosidase Activity

Using 96 well plates, 50 μ l of each sample was combined with 50 μ l of 2X Assay buffer (Promega, Australia) containing 120mM Na₂HPO₄, 80mM NaH₂PO₄, 2mM MgCl₂, 100mM β -Mecarptoethanol and 1.33mg/ml O-nitrophenyl-beta-D-galactopyranoside (ONPG) and incubated for between 30 minutes and 3 hours to allow the colour reaction

to develop. Following the incubation period, β -Galactosidase activity was measured using a microplate reader (Biorad 3550-UV) to determine the absorbance of each sample at 405nm.

Luciferase Activity

Immediately following the addition of 100 μ l of Luciferase Assay Reagent (Promega, Australia) to 20 μ l of sample, luminescence was read using a Turner Designs TD20/20 luminometer. For each sample, the luminometer performed a 2 second measurement delay followed by a luminescence measurement after 10 seconds. Background luminescence was also determined by measuring the luciferase activity of cell lysates containing no DNA.

3.2.2.2 Statistical analyses

Statistical analysis of transfection data was performed using a 2 sample T test in MINITAB. The difference in the data was deemed significant if the p value obtained was less than 0.05.

3.3 RESULTS

3.3.1 Preparation of Nuclear Extracts

In preliminary experiments, different methods for the preparation of cell nuclear extracts were examined to determine which method resulted in a better yield of nuclear proteins. One of these methods, derived from Schreiber *et al.*, (1989) resulted in a total protein yield of 166 μ g from 1×10^7 cells, while a method, derived from Andrews and Faller (1991) resulted in a total nuclear protein yield of 260 μ g from the same number of cells. Not only was this higher than the yield from the Schreiber based method but the protein was recovered in a much smaller volume. It is important to have a concentrated nuclear protein extract for EMSA because each EMSA reaction only allowed for a maximum volume of 4 μ l of nuclear extract and typically 2-10 μ g of nuclear extract are

used in each reaction. The major differences between the two methods was that in the Andrews and Faller based method a larger number of different protease inhibitors were used and the extraction buffer contained glycerol. It is important that nuclear extract preparations be stored at or below -70°C to prevent degradation of the proteins by proteases. The presence of glycerol in the extraction buffer helps prevent damage to the nuclear protein upon freezing. A further difference was that the nuclear extracts from the Andrews and Faller based method were dialysed after isolation in order to remove salts from the extract, which is desirable since excess salt can inhibit the binding of protein to the DNA in the EMSA. The method based on Andrews and Faller (1991) was used for the nuclear extract preparations from each of the K562 derived MDR cell lines, however in later experiments the dialysis step was omitted. Trial experiments demonstrated that there was no difference between the binding of dialysed and undialysed extracts and omitting the dialysis step aided the reduction of protein degradation in the sample.

3.3.2 Y-box binding factor levels in K562 and MDR sublines

The Y-box has previously been implicated in *MDR1* up-regulation, therefore oligonucleotides were designed to produce a 30bp double stranded radiolabelled DNA fragment encompassing the *MDR1* promoter Y-box (as shown in section 3.2.1.2) for use in EMSAs to investigate levels of Y box binding factors. Figure 3.1 demonstrates the specificity of binding to the Y-box probe as well as to a control USF binding probe used to normalise the levels of binding factors in different preparations of nuclear extracts. As shown in Figure 3.1 two major high molecular weight complexes (A and B) and several minor low molecular weight complexes were formed with the Y-Box. The binding of most of these complexes was specific as shown by the significant reduction in binding to labelled probe caused by the presence of competing unlabelled self probe (Figure 3.1a) but not by competition with a non-self (USF) competitor (Figure 3.1b). Two specific complexes (I and II) also bound to the USF probe but only complex I appeared specific (Figure 3.1c).

The levels of Y box binding factor in the various MDR cell lines before and after induction with P-gp substrates were then examined (shown in Figure 3.2). Comparisons

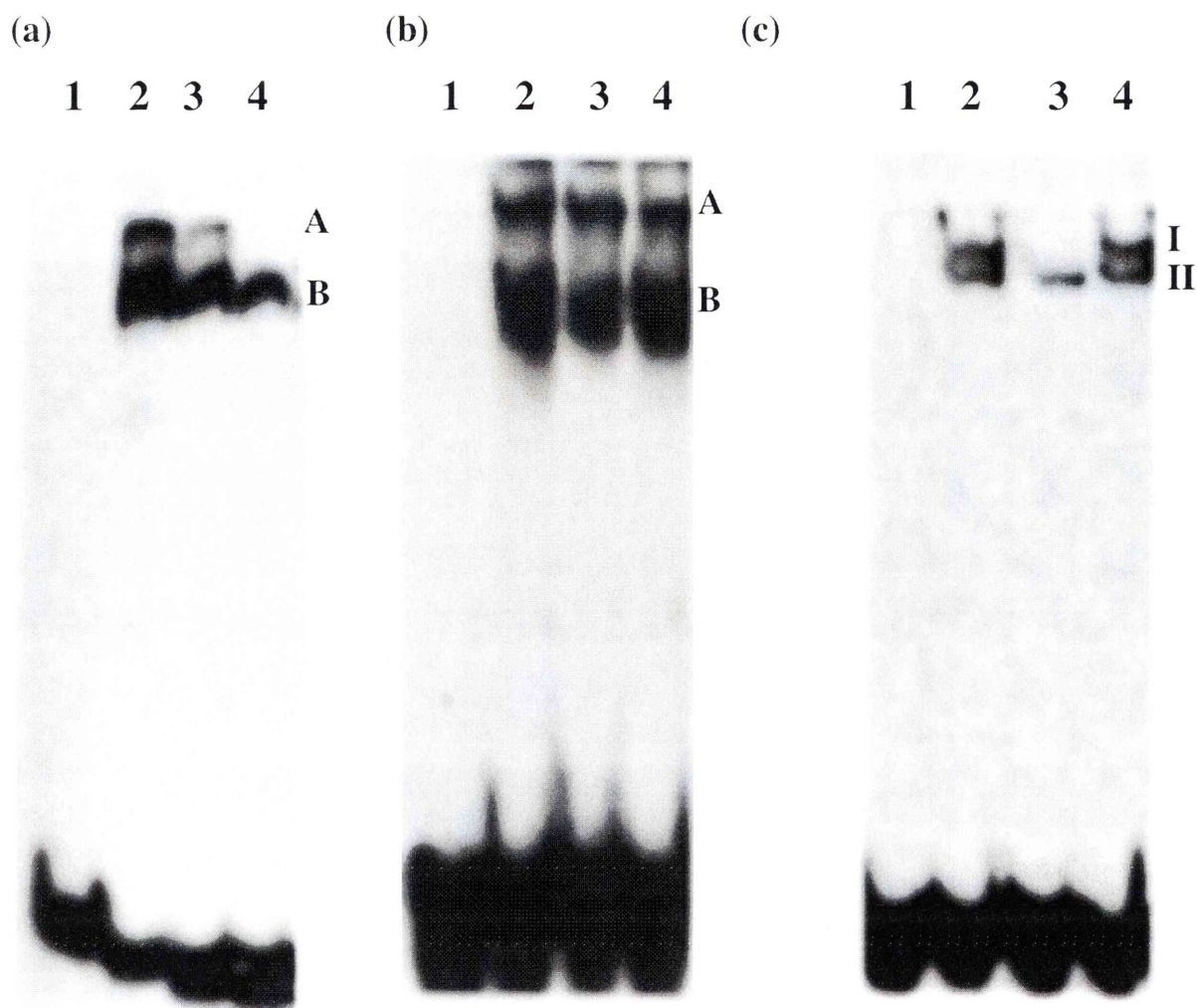


Figure 3.1 EMSA for (a) Y-box binding probe where lane 1 contains no extract and lanes 2-4 contain $5\mu\text{g}$ of nuclear extract from K/DNR cells, and lanes 3 and 4 contain a 50x and 200x fold concentration of unlabelled self competitor. (b) Y-box binding factor where lane 1 contains no extract and lanes 2-4 contain $5\mu\text{g}$ of nuclear extract from K562, and 3 and 4 contain a 50x and 200x fold concentration of unlabelled non-self (USF) competitor binding DNA. (c) USF binding factor where lane 1 contains no extract and lanes 2-4 contain $1\mu\text{g}$ of nuclear extract from K562, and lanes 3 and 4 contain 50x fold concentration of unlabelled self (USF) and non-self (Y-Box) competitor binding DNA respectively.

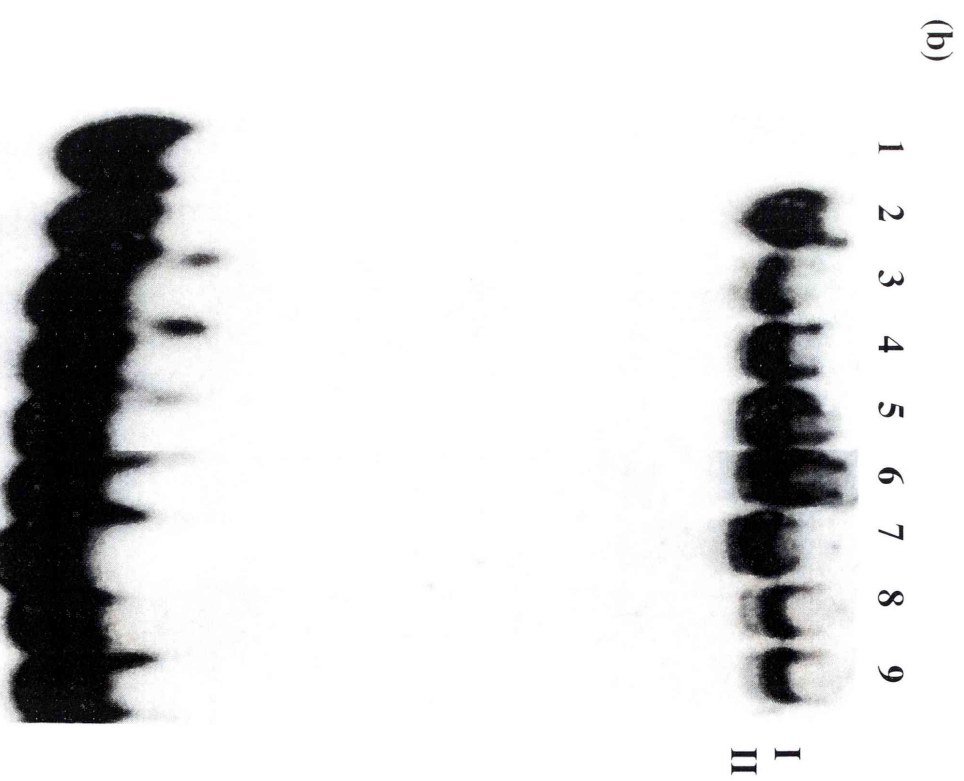
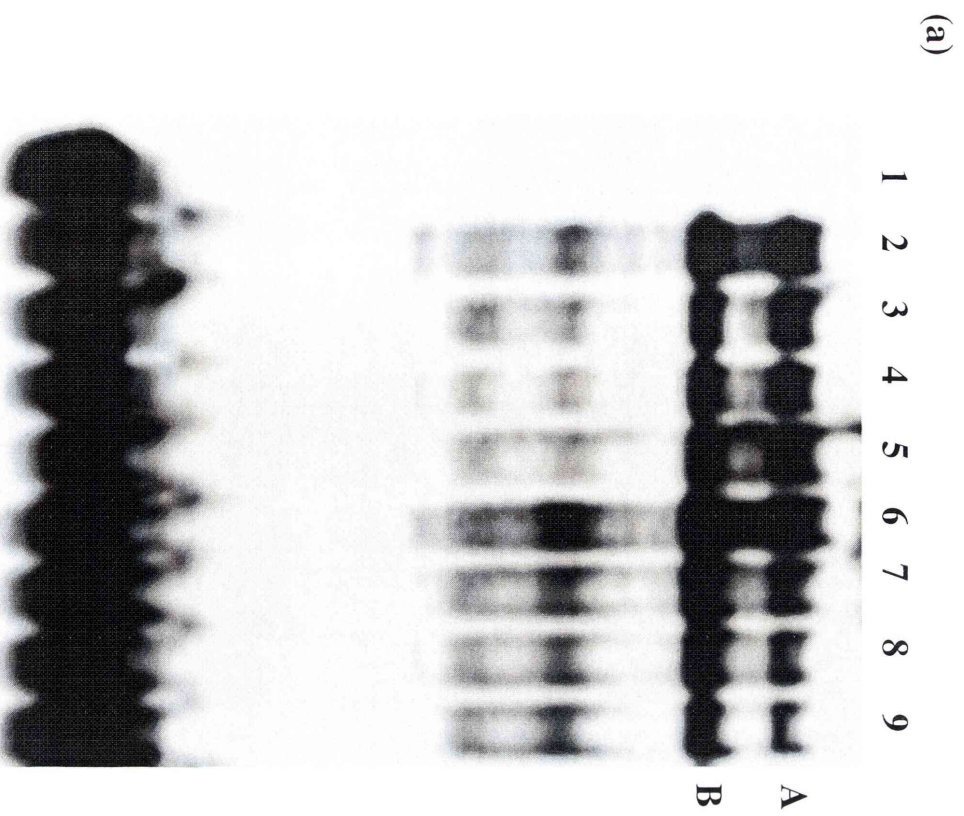


Figure 3.2 EMSAs with (a) Y-box binding probe where lane 1 contains no extract and lanes 2-9 contain 5 μ g of nuclear extract from K562 (lane 2), K562 treated (lane 3) K/EC32 lane 4), K/EC32 treated (lane 5) K/DNR (lane 6) K/DNR treated (lane 7) K/EPR (lane 8) and K/EPR treated (lane 9) and (b) USF binding probe where lanes 1-9 are as above except that 1 μ g nuclear extract was used in EMSAs.

were made between cells grown in drug free culture for 28 weeks (K562 and K/EC32) or 17 weeks (K/DNR and K/EPR) and MDR cells whose drug resistance levels had been increased by treatment with their original selecting drug concentration for 3 days (see section 2.2.7) as well as K562 cells that had been treated with 5ng/ml epirubicin, albeit with no effect on drug sensitivity. Figure 3.2a shows results of an EMSA using nuclear extracts from the above cell lines and the Y-box probe and Figure 3.2b shows the results of an EMSA performed with the same extract preparations and the USF binding probe. Densitometry analysis of the major specific complexes A and B formed with the Y-box and complexes I and II formed with the USF binding sequences was carried out and normalised levels of Y-box binding factor were expressed as a ratio of Y-box sequence binding to USF sequence binding (Table 3.1). As indicated there appeared to be no major apparent differences in box binding factor between extracts from K562 cells and derived MDR sublines, either before or after induction by P-gp substrate drug. Repeat experiments confirmed this finding, thus the increased drug resistance of these cell lines did not correlate with gross increases in absolute amounts of Y box binding protein.

3.3.3 Identification of Y-box binding factor

The identity of the Y-box binding factor is subject to some dispute with some investigators reporting that the factor NF-Y binds to the Y-box whilst others that the factor Y-B1 is the Y box binding factor. Y-B1 also reportedly binds preferentially to single stranded DNA (Hasegawa *et al.*, 1991; Kolluri *et al.*, 1992; Tafuri and Wolffe, 1992; MacDonald *et al.*, 1995; Bayarsaihan *et al.*, 1996; Mertens *et al.*, 1997; Ohmori *et al.*, 1996; Kloks *et al.*, 2002). Therefore to test for the presence of YB-1 in nuclear extracts from K562 derived cells a single stranded Y-box oligonucleotide was used as binding probe in EMSAs with nuclear extracts from K562, K/EPR, and K/EPR cells after 16 hour treatment with 160ng/ml epirubicin (see section 2.2.8). As shown in Figure 3.3i(a) a single protein:DNA complex was bound to the 14mer Y-box probe, however competition studies revealed this complex was non-specific. That is while its formation was significantly or completely reduced by the addition of 100-fold molar excess unlabelled self ssYB-1 oligonucleotide, it was also significantly reduced with the addition of either unrelated unlabelled single stranded USF binding sequence or double

Table 3.1 Normalised Y-box binding factor levels in K562 MDR sublines

| | Volume Y-box complexes | Volume USF complex | Ratio Y-box : USF |
|------------------------|-----------------------------------|-----------------------------------|------------------------------|
| K562 | 35 | 34 | 1.0 |
| K562 drug treated | 29 | 24 | 1.2 |
| K/EC32 | 33 | 29 | 1,1 |
| K/EC32 drug treated | 38 | 38 | 1.0 |
| K/DNR | 38 | 39 | 1.0 |
| K/DNR drug treated | 30 | 34 | 0.9 |
| K/EPR | 25 | 23 | 1.1 |
| K/EPR drug treated | 20 | 21 | 1.0 |

stranded USF binding sequence. These results were obtained reproducibly and thus suggests that the Y-B1 protein is not found in nuclear extracts from K562 and K/EPR cells.

To further investigate the identity of the factor binding to the Y-box in the *MDR1* promoter in our cells, the effect of an anti-NF-YA antibody on the Y-box binding complex formed with K/EPR nuclear extracts was tested. As shown in Figure 3.3i(b) two DNA:protein complexes were formed with the Y-box probe (lane 2) and a supershift in the migration of both these complexes to a position near the wells of the gel was seen after incubation with the anti-NF-YA antibody. No shifting of complexes formed with USF binding sequence occurred. This result is reproducible in K562 cell extracts and extracts from drug treated K/EPR cells, as is seen by the shift of all specific DNA:protein complexes formed with the Y-box probe after incubation with the anti-NF-YA antibody in figure 3.3(ii). Therefore these results show that the complexes formed *in vitro* with nuclear extracts from the K562 and K562 MDR subline K/EPR and the Y-box are characteristic of NF-Y.

3.3.4 -55GC box binding factor levels in K562 and MDR sublines

To investigate the role of factors binding to the -55GC box of the *MDR1* promoter a binding probe encompassing this region was incubated with nuclear extracts from K562 cells and untreated and drug treated K/EPR sublines. Figure 3.4 shows an example of the results of an EMSA using 3 μ g of nuclear extract from K562 (lane 6), K/EPR (lane 5) and K/EPR pre-treated for 16 hours with 160ng/ml of epirubicin (lane 4). Five complexes were seen with the -55GC box sequence with complexes I, IV and V being specific since competition with a 100-fold excess of unlabelled -55GC box binding sequence reduced binding dramatically, whereas a 100-fold excess of unrelated USF binding sequence had no effect on complex binding. One major and two minor complexes were formed with the USF binding sequence, however only complex A and C were found to be specific. Densitometry analysis was carried out on specific complexes from the -55GC box and USF binding sequences, with USF binding used to normalise levels of -55GC box binding factor. Normalised levels were expressed as a

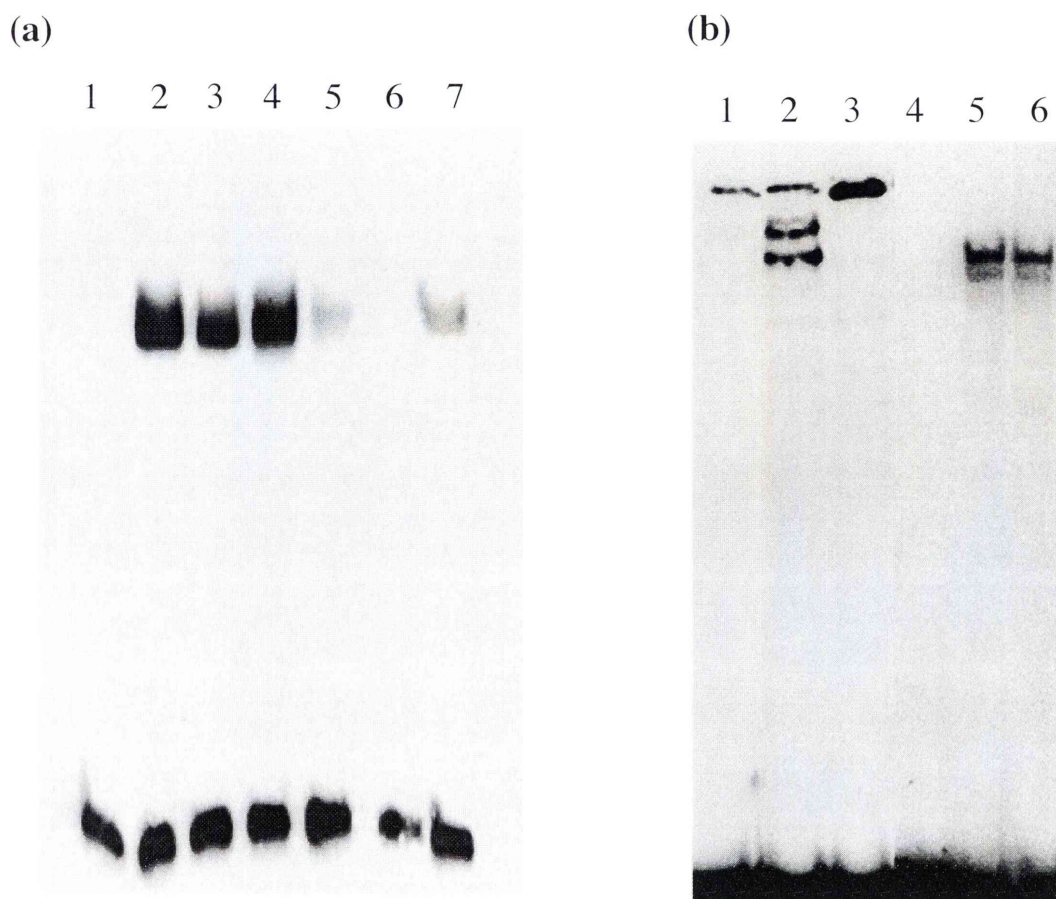


Figure 3.3 (i) (a) EMSA using single stranded YB-1 binding oligonucleotide where lane 1 contains no extract and lanes 2-4 contain $3\mu\text{g}$ of nuclear extract from K562 (lane 2), K/EPR (lane 3), K/EPR treated (lane 4). Lane 5 has a 100-fold excess of single stranded self competitor DNA, lane 6 a 100-fold excess of unrelated single stranded and lane 7 a 100-fold excess of unrelated double stranded USF competitor DNA.

(b) Supersifting of Y-box binding factor with anti-NF-YA antibody for Y-box (lanes 1-3) and USF binding sequence (lanes 4-6), where lanes 1 and 4 contain no extract, lanes 2 and 5 contain $3\mu\text{g}$ K/EPR nuclear extract and lanes 3 and 6 contain $3\mu\text{g}$ K/EPR extract incubated with anti-NF-YA.

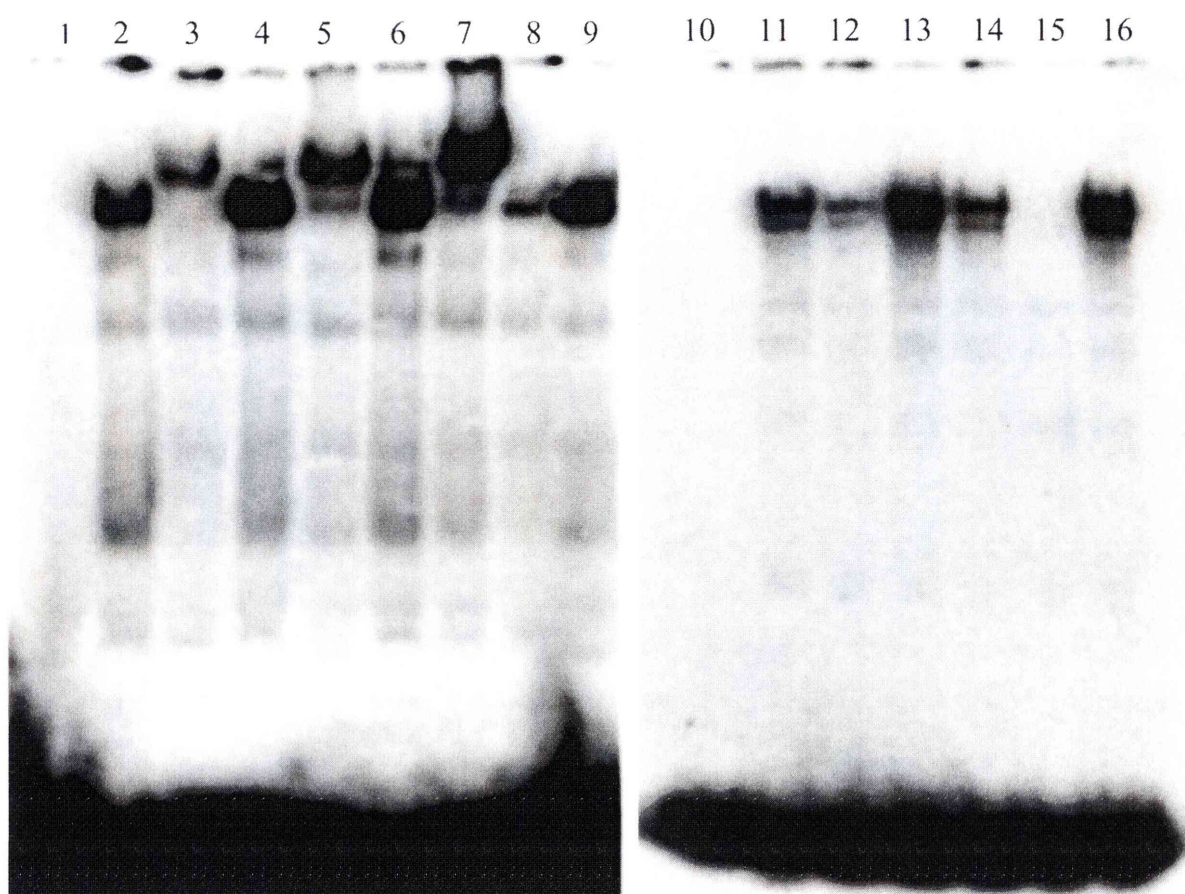


Figure 3.3 (ii) EMSA for supershifting of Y-box binding factor with anti-NF-YA antibody for Y-box (lanes 1-9) and USF binding sequence (lanes 10-16), where lanes 1 and 10 contain no extract, lanes 2,3 and 11 contain 3ug of K562 nuclear extract, lanes 4, 5, 8, 9, 12, 14, 15 and 16 contain 3ug K/EPR nuclear extract and lanes 6, 7 and 13 contain 3ug of nuclear extract from K/EPR cells treated for 16hours with 160ng/ml of epirubicin. The nuclear extracts in lanes 3, 5, 7 and 14 were incubated with anti-NF-YA. Lanes 8 and 15 have a 100-fold excess of self competitor DNA and Lanes 9 and 16 have a 100-fold excess of unrelated competitor DNA.

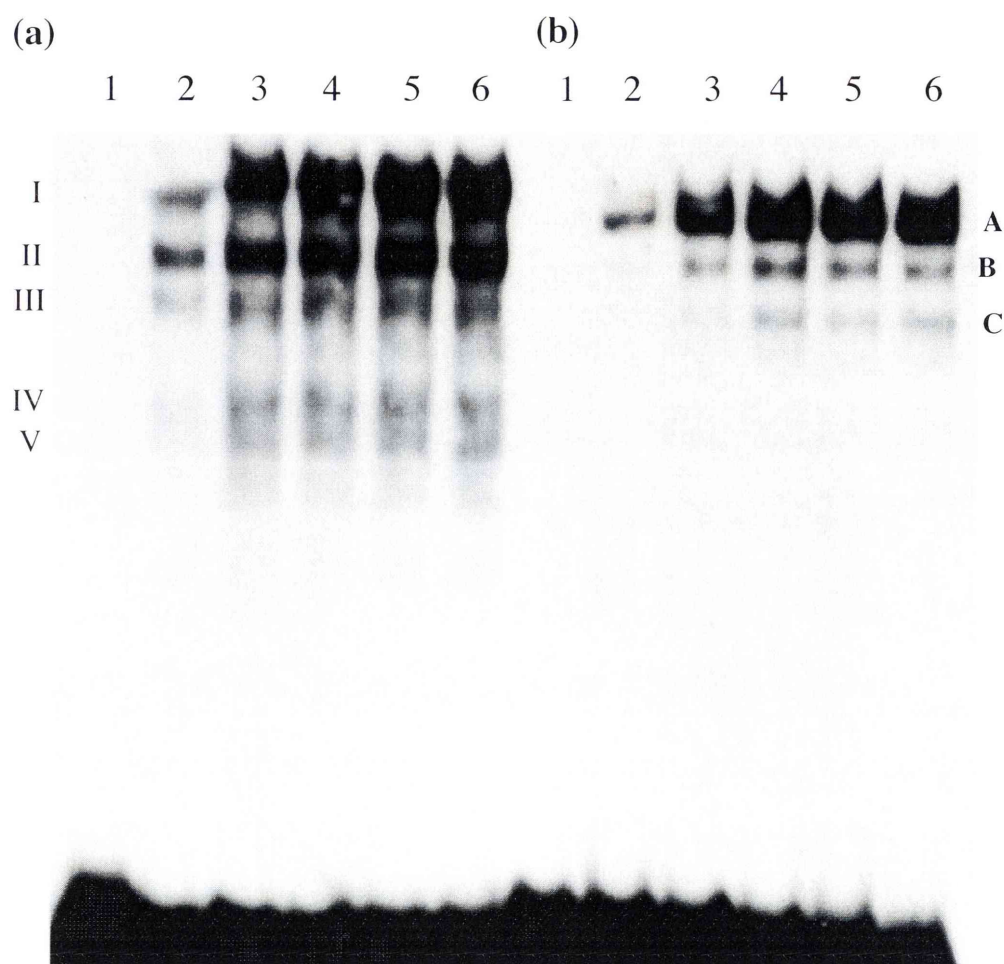


Figure 3.4 EMSA for (a) -55GC box and (b) USF where lane 1 contains no extract and lanes 2-6 contain $3\mu\text{g}$ of nuclear extract from K562 (lane 6), K/EPR (lane 5), K/EPR treated (lane 4). Lane 2 has a 100-fold excess of self competitor DNA and nuclear extract from K/EPR treated cells. Lane 3 had a 100-fold excess of unrelated competitor DNA and nuclear extract from K/EPR treated cells. For the -55CG box, binding complexes are labelled I, II, III, IV and V. For the USF binding sequence complexes are labelled A, B, and C.

ratio of –55GC box binding factor to USF binding. As shown in Table 3.2 the levels of –55GC box binding factor appeared similar are in K562 and K/EPR cells. Moreover the drug treatment that resulted in induced MDR levels showed no effect binding levels. These results were reproducible in numerous subsequent experiments ($n > 10$).

3.3.5 Transient Transfection of *MDR1* Promoter

The EMSAs discussed above were used to compare absolute levels of binding factors to the Y-box and the –55GC box in the *MDR1* promoter regions in the K562 and K/EPR cells. However these are *in vitro* experiments and the binding or transactivation ability of transcription factors *in vivo* may be influenced by protein-protein interactions or specific modification of binding factors. Therefore in order to test the importance of these two regions in a more *in vivo* model, reporter gene studies were carried out. K562 and K/EPR cells were transfected with a luciferase reporter gene system previously designed in the laboratory to contain an *MDR1* promoter region (–88/+76), referred to as *Luc1*, subcloned into the upstream promoter site of the luciferase reporter vector pGL3-Basic (Cassano, Honours thesis, 2000).

The –88/+76 region was chosen since previous transfection studies have shown that *MDR1* promoter activity is not increased with the inclusion of regions beyond –88, (Cornwell and Smith, 1993; Goldsmith *et al.*, 1993). Further studies have indicated that maximum promoter activity is achieved by terminating the promoter at +76 (Lau *et al.*, 1998). Most importantly, this *Luc1* construct encompasses both the –55GC box and the Y-box. CMV- β -galactosidase was co-transfected with *Luc1* to normalise for transfection efficiency. CMV- β -galactosidase was chosen as its promoter does not contain any Sp1 binding sites, and therefore will not compete for possible Sp1 binding sites in the – 55GC box in the *Luc1* construct. As shown in Figure 3.5, the transfected – 88/+76 *MDR1 Luc1* promoter was as active in K562 cells as it was in MDR K/EPR cells. In fact it appeared slightly less active in the MDR K/EPR cell line, although statistical analysis revealed no significant difference between the activity in these two cell lines. Thus the absence of endogenous *MDR1* mRNA in K562 cells was not due to inactivity of, or inability to bind by essential transcription factors.

Table 3.2 –55GC box binding factor levels* in K562, K/EPR and K/EPR drug treated cells

| K562 | K/EPR | K/EPR drug treated |
|-------------|--------------|---------------------------|
| 2.58 | 2.65 | 2.48 |

* levels are normalised relative to USF levels in each cell line as follows,

$$\frac{\left(\frac{\text{Total -55GC box bound complexes I, IV and V}}{\text{Total -55GC box DNA (bound+unbound)}} \right)}{\left(\frac{\text{Total USF binding complex A and C}}{\text{Total USF DNA (bound+unbound)}} \right)}$$

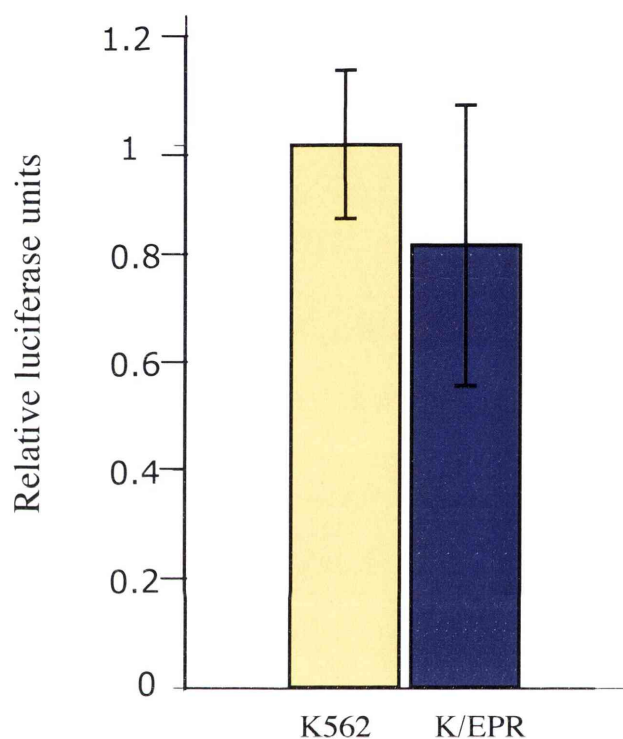


Figure 3.5 Relative *MDR1* promoter activity in K562 (■) and K/EPR cells (■). Individual *MDR1* promoter activities are standardised by dividing luciferase units by co-transfected β -galactosidase units. Activity in K/EPR cells is expressed relative to that in K562 cells determined in the same experiment. The average and standard deviation of 9 separate determinations (triplicate wells from 3 experiments) are shown for each cell line.

Previous transfection experiments in this laboratory have indicated negative regulation when the +283/+455 region of the *MDR1* promoter was cloned downstream of the *Luc* gene in the *Luc1* construct and transfected in an MDR cell line derived from the HL60 cell line (Byrnes, unpublished results). In an attempt to localise further the negative regulatory element, the +283/+455 region was further subdivided into 2 additional constructs: *Luc3AB* and *Luc3BC* which contained respectively the +283/+392 and +329/+455 regions cloned into a site downstream of the *luc* gene in the *Luc1* construct (Michelle Byrne, Honours thesis 2001). To test if these regions played any role in *MDR1* induction the K/EPR cells were treated with and without 160ng/ml epirubicin for 16 hrs, then washed and transfected with either *Luc1*, *Luc3AB* or *Luc3BC*, plus CMV- β -Galactosidase DNA. Luciferase activity was measured after 48 hrs and normalised with respect to β -galactosidase activity. Empty vector transfections were also carried using the pGL3-Basic vector as a control.

Figure 3.6 shows the normalised relative light units for a typical transfection of K/EPR cells with either the PGL3-basic vector, *Luc1*, *Luc3AB* or *Luc3BC* constructs before and after drug induction of MDR. It is clear from this that epirubicin treatment had no effect on *MDR1* promoter activity with any of the constructs tested, (where *Luc1* $p=0.613$, *Luc3AB* $p=0.742$ and *Luc3BC* $p=0.890$ when comparing treated versus untreated cells). Thus the induction of *MDR1* mRNA produced upon exposure of the K/EPR cells to drug was not accompanied by a change in the activity of, or binding ability of any transcription factors that may bind to these putative repressor elements.

3.3.6 Repressor Binding Protein levels in K562 and K/EPR cell lines

A previous study has suggested the presence of a CAAT-containing negative response element (NRE) at approx – 120 to –112 of the *MDR1* promoter (Ogura *et al.*, 1992; Ogretman and Safa, 1999). To determine the relative levels of a possible repressor protein binding to this region, nuclear extracts from K562 cells, K/EPR cells and K/EPR cells treated with 160ng/ml of epirubicin for 16 hours, were incubated with a DNA binding probe encompassing –129 to –106 bp. As shown in Figure 3.7, a single specific DNA protein complex was obtained for both the K562 and K/EPR untreated and epirubicin treated cell lines. Densitometry was performed on the binding complexes

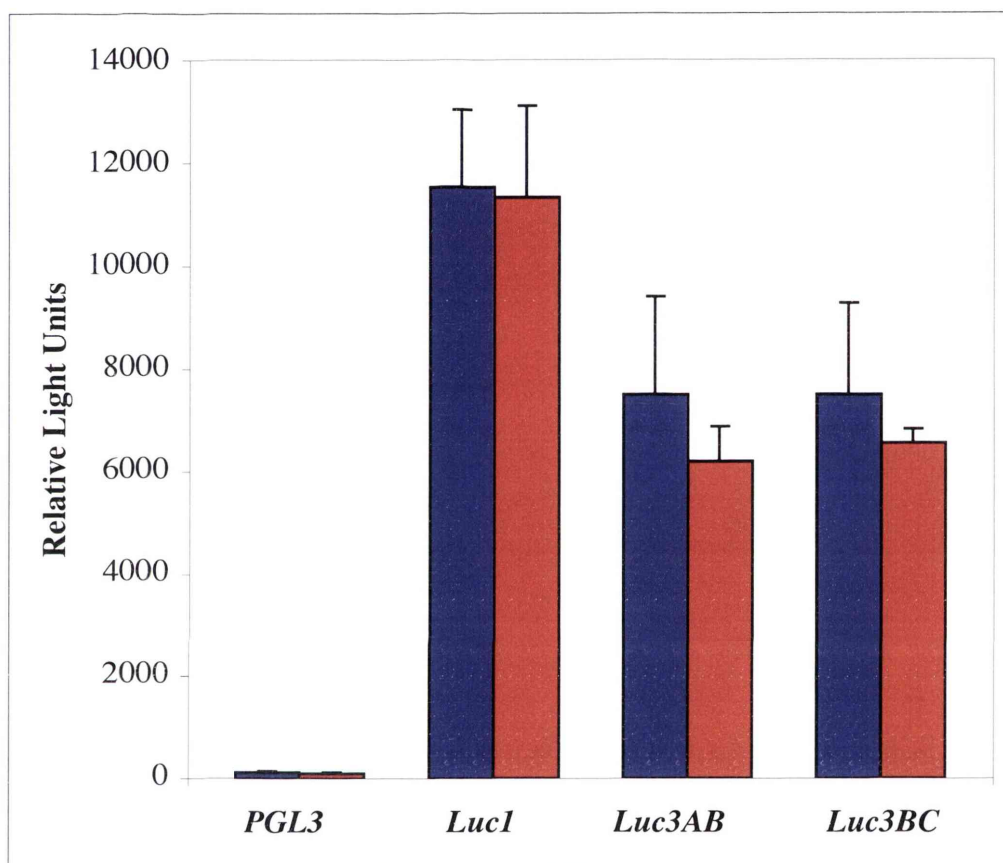


Figure 3.6 *MDR1* promoter activity in K/EPR untreated (■) and K/EPR treated cells (■). Individual *Luc1*, *Luc3AB* and *Luc3BC* *MDR1* promoter activities are standardised by dividing luciferase units by co-transfected β -galactosidase units. The average and standard deviation of 3 separate determinants are shown for each cell line.

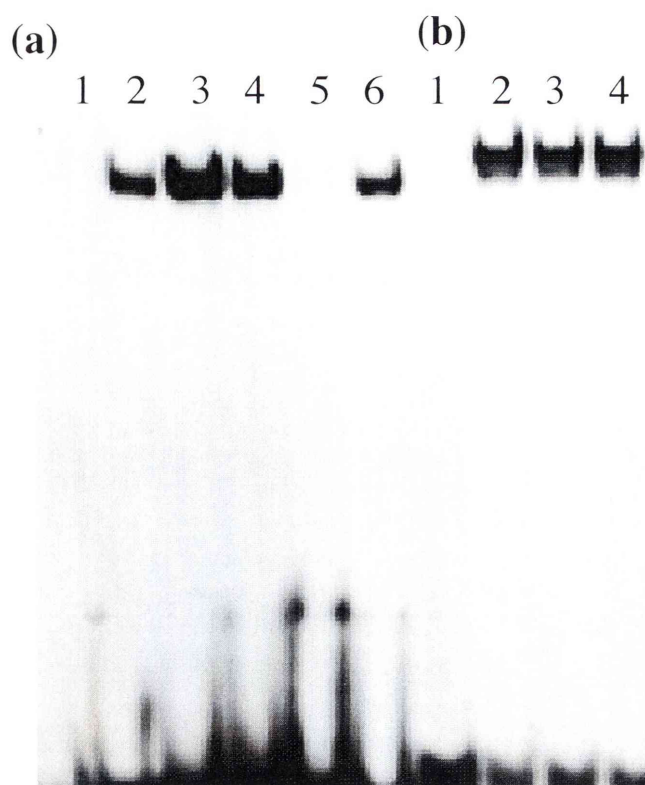


Figure 3.7 EMSA for (a) NRE binding factor and (b) USF using no extract (lane 1), 3 μ g of nuclear extract from K562 (lane 2), K/EPR (lane 3) and K/EPR treated (lane 4). Lane 5 has a 100-fold excess of self competitor DNA and 3 μ g of K/EPR treated nuclear extract lane 6 has a 100-fold excess of unrelated competitor DNA and 3 μ g of K/EPR treated nuclear extract.

Table 3.3 NRE binding factor levels* in K562, K/EPR and K/EPR drug treated cells

| K562 | K/EPR | K/EPR drug treated |
|------|-------|-----------------------|
| 0.87 | 1.00 | 0.84 |

* levels are normalised relative to USF levels and calculated as follows,

$$\left(\frac{\text{Total of NRE bound complex}}{\text{Total NRE DNA (bound+unbound)}} \right)$$

$$\left(\frac{\text{Total of USF binding complex}}{\text{Total USF DNA (bound+unbound)}} \right)$$

and the NRE binding complex was normalised with USF binding complex (Table 3.3). These results indicate that the levels of the putative repressor in K562, K/EPR untreated and epirubicin treated MDR cells were similar in all extracts, suggesting that the absence of *MDR1* expression in K562 cells is not due to the presence of the negative repressor in these cells only.

3.4 SUMMARY OF RESULTS

In view of the controversy over the role of Y-box binding proteins we investigated the amounts and identity of proteins able to bind to the Y-box in K562 MDR cells and whether drug induction was mediated by changes in Y-box binding protein. Supershift experiments identified the Y-box binding protein in our cell lines to be NF-Y and not YB-1. We found similar levels of NF-Y in the MDR cells and the relatively drug sensitive parental K562 cells. Therefore, whilst NF-Y may be essential for *MDR1* activity, its presence is not sufficient to ensure P-gp expression in K562 cells. Moreover the difference between K562 and its MDR cell lines is not related to differences in absolute amounts of available nuclear NF-Y factor.

A single stranded YB-1 binding sequence was also included in the EMSA studies on the Y-box as YB-1 has been shown to preferentially bind to single stranded DNA (Hasegawa *et al.*, 1991; Kolluri *et al.*, 1992; Tafuri and Wolffe, 1992; MacDonald *et al.*, 1995; Bayarsaihan *et al.*, 1996; Mertens *et al.*, 1997; Ohmori *et al.*, 1996; Kloks *et al.*, 2002). However we found no specific protein binding to the single stranded oligonucleotide, thus strengthening the argument for the identification of NF-Y as the protein binding to Y-box in the *MDR1* promoter of K562 derived cell lines. It was also questioned whether induced MDR levels and upregulation of *MDR1* transcription upon drug treatment coincided with increased NF-Y levels in the cells, however Y-box binding factor levels were not effected by short term drug exposure. Similar to NF-Y, levels of binding to the -55GC box also did not differ between K562 and the K/EPR MDR cell line and did not change after drug exposure. Levels of protein complex binding to an NRE, containing a CAAT-like motif at -118 to -113, were also found to

be present in similar levels in both K562 and K/EPR cells suggesting that the negative regulation of *MDR1* expression in K562 cells, compared to K/EPR cells, is not caused by differences in absolute amounts of the factor binding to the -118/-113 NRE.

EMSAs are an *in vitro* experiment and thus do not take into account the possibility that the binding or transactivation ability of the transcription factors *in vivo* may be influenced by protein-protein interactions or specific modification of bound factors. However the finding that the -88/+76 *MDR1* promoter reporter, which contains the Y-box and the GC box, is equally effective in driving reporter gene expression in K562 cells as it is in K/EPR cells, suggests that such factors are not the cause of the differences in resistance and *MDR1* mRNA levels between the K562 and K/EPR *MDR* cell lines. This conclusion is supported by the finding that -88/+76 *MDR1* promoter driven reporter gene expression was not effected by drug induction of *MDR* in the K/EPR cell line. Transfection of the K/EPR cell line with constructs encompassing +283/+392 or +329/+455 regions of the *MDR1* promoter, confirmed the presence of a possible negative regulatory element in this region. However, repression by these regions was also not effected by drug induction of K/EPR cells.

Thus our finding suggest that changes in the availability of factors binding to the -55GC box, Y-box and -118/-113 NRE cannot be responsible for P-gp up-regulation in K562 derived *MDR* cell lines.

CHAPTER 4

MDR1 Promoter Methylation Studies

4.1 INTRODUCTION

The results described to date indicate that increased nuclear levels of Y-box or –55GC box binding proteins are not responsible for the differences in *MDR1* activation in K562 and derived MDR sublines.

Since repression of *MDR1* transcription may also result from hypermethylation of its promoter (Desiderato, 1997; Kantharidis *et al.*, 1997) it was hypothesised that the absence of P-gp expression in the K562 cells was due to methylation. Therefore bisulphite genomic sequencing was used to determine the methylation status of CpG dinucleotides in the *MDR1* promoter of K562 and K/EPR cells. The technique, shown schematically in figure 4.1, exploits the ability of sodium bisulphate to selectively and efficiently deaminate cytosine residues into uracil whilst 5-methylcytosines do not react and thus remain unconverted as cytosines (Clarke *et al.*, 1994). Upon subsequent polymerase chain reaction (PCR) using bisulphate deaminated DNA strand specific primers, the converted uracil residues amplify as thymines whereas 5-methylcytosines remain as cytosines. Products can then be subcloned and sequenced and the methylation status of the CpG dinucleotides can be assessed by comparison of the converted sequence with the original unconverted sequence. In this study, the efficiency of the bisulphite conversion of the DNA was increased by the addition of urea to the bisulphite reaction (Paulin *et al.*, 1998).

4.2 MATERIALS AND METHODS

4.2.1 Genomic Sequencing

4.2.1.1 Bisulphite Conversion of Genomic DNA

Genomic DNA was extracted from K562 and K/EPR cells at approximately 70% confluent growth as described in section 2.2.5. The DNA was restriction endonuclease digested with EcoR1, an enzyme which does not cut the *MDR1* promoter region of interest. EcoR1 digests typically contained 1 – 10 μ g of genomic DNA and between 5 – 10 units of enzyme per μ g of target DNA. Digests were performed in the buffer and at the temperature recommended by the supplier and supplemented with 100 μ g/ml acetylated BSA. Reactions were incubated for at least 1 hour, occasionally overnight. After incubation, 0.2 – 1 μ g of the DNA was run in a 1% agarose gel for an indication of digestion completion.

Bisulphite conversion of genomic DNA was based upon the method described by Clark *et al.*, (1994). The procedure involves the modification of unmethylated cytosine residues to uracil under conditions where methylated cytosines remain unchanged. Briefly, 1 μ g of EcoR1 digested genomic DNA was denatured by addition of freshly prepared 3M NaOH to a final concentration of 0.3M and incubated at 37°C for 15 minutes. The denatured DNA was then treated by addition of freshly prepared 2.0M sodium bisulphite (BDH), pH5.0 (pH with 10M NaOH) to a final concentration of 1.7M and freshly prepared 10.0mM hydroquinone (BDH) to a final concentration of 0.5mM. Urea at a final concentration of 5.36M was also added in the DNA bisulphite conversion, as this has been shown to enhance the conversion (Paulin *et al.*, 1998). The solution was overlaid with mineral oil and incubated at 55°C for 3 hours followed by 94°C for 3 minutes and this cycle was repeated five times. Following incubation the DNA was purified using Bresa clean (Genesearch) and incubated at 37°C for 15 minutes with freshly prepared NaOH to a final concentration of 3.0M to neutralise the sample, and the DNA was ethanol precipitated, dried, resuspended in an appropriate volume of sterile water and stored at –20°C.

4.2.1.2 PCR of converted genomic DNA

Bisulphite converted genomic DNA was amplified by the polymerase chain reaction (PCR). The target region was amplified by PCR using nested primers comprising the two forward primers :

M1: 5' TATTAAATAAAGGATGAATAGATGTAATTTAG 3';

M3: 5' TTGTTAAGTATGTTGAAGAAAGATTATTGT 3'

and the two reverse primers:

M2: CAACATATCATTTATTTCAAAACTAAAAAC 3';

M4: 5' AAAACAACTAATTACCTTTTATTATTCAAT 3' (Invitrogen).

PCR reagent concentrations were initially varied to determine optimum conditions for amplification. The final optimal conditions used for each sample are given below. PCR reactions were carried out in 25µl total volume reactions containing 37mM Tris-Cl pH 8.8, 16.6mM (NH₄)₂SO₄, 0.45% TritonX-100, 0.2mg/ml gelatin, 1.5mM MgCl₂, 200µM dNTPs, 1µM primers, 0.6 units Taq polymerase (Applied Biosystems) and 25-50ng converted DNA using the following PCR parameters {94°C /1min, 50°C /2min, 72°C /3min} x 5 cycles , {94°C /0.5min, 50°C /2min, 72°C /1.5min} x 30 cycles and {72°C /6min} x 1 cycle. PCR products were run in a 1.5% agarose 1X TAE gels along with pGEM DNA size markers (Promega), then excised and purified using the Bresa-clean method. Purified PCR products were ligated into T-tailed pGEM-T vector (Promega) using optimised vector:insert molar ratios and subcloned into calcium competent *E.coli* DH5α cells.

4.2.1.3 Subcloning of PCR Products

Preparation and Ligation of Insert DNA to pGEM-T Vector

PCR product obtained from the amplification of sodium bisulphite converted DNA were run in 1.5% agarose 1X TAE gels along with pGEM DNA size markers, then excised and purified using the Bresa-clean method, prior to ligation to pGEM-T vector (Promega). Ligation reactions were performed overnight at 4°C with a vector: insert molar ratio of 1:1 in a 10µl reaction containing 1x T4 DNA Ligase buffer (Promega)

and 1 unit of T4 DNA Ligase (Promega). This was then subcloned into *E. coli* DH5 α cells.

Preparation and Transformation of Competent Cells

E. coli (DH5 α) cells were streaked from glycerol stocks onto an LB agar plate and incubated overnight at 37°C. A single colony was chosen, inoculated into 75mls of LB medium and grown overnight at 37°C, 300rpm. The following morning the culture was diluted 1/40 - 1/100 in LB medium and grown as before until the OD₆₀₀ was approximately 0.15. All subsequent steps were performed on ice to ensure that competent cells with high transformation efficiency were obtained. The culture was incubated on ice 10 minutes prior to centrifugation for 7 minutes at 3000g, 4°C. Excess media was drained off and the pellet of cells was gently resuspended in 1/5th volume of ice cold Calcium buffer (60mM CaCl₂, 15%v/v glycerol/10mM PIPES, pH 7) and respun as above. The supernatant was again discarded and the cells gently resuspended in 1/5th volume of ice cold Calcium buffer and allowed to stand on ice for at least 30 minutes. The cells were pelleted at 3000g, 4°C, and resuspended in 1/25th volume Calcium buffer. 210 μ l aliquots were dispensed into pre-chilled microcentrifuge tubes, snapped frozen on dry ice and stored at -80°C until required.

The required amount of DNA was added to 0.2ml of competent cells and incubated on ice for 30 minutes. Competent cell/DNA mixtures were heat shocked at 42°C for exactly 90 seconds and immediately returned to the ice and allowed to chill for 1-2 minutes. 800 μ l of LB medium was added to the mixture and placed in a rotary shaker at 300rpm, 37°C for 45 minutes to allow the bacteria to express the plasmid genes. Aliquots (100 μ l and 200 μ l) of the transformed cells were transferred to two agar plates containing ampicillin (100 μ g/ml) spread evenly over the surface of the plate and incubated overnight at 37°C.

Identification of Recombinants

Putative recombinant clones were amplified by picking white colonies and growing these in 2.5mls of LB broth overnight at 37°C/300r.p.m. Cells were collected by centrifugation at 11600g for 3 minutes and lysed by the addition of 300 μ l of TENS buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1 N NaOH, 0.5% SDS)

followed by a brief vortex. The lysate was neutralised by addition of 150µl of 3.0M sodium acetate (pH 5.2) and given a brief vortex. Cellular debris and nuclear DNA was removed by centrifugation at 11600g for 2 minutes. The supernatant was extracted with an equal volume of phenol (Tris buffered, pH 7): chloroform: isoamyl alcohol (25:24:1,v:v:v). The mixture was centrifuged at 11600g for 5 minutes at RT, then the aqueous phase was removed and extracted with an equal volume of chloroform: isoamyl alcohol mixture (24:1, v:v). This was then centrifuged at 11600g for 5 minutes at RT and the DNA was then ethanol precipitated from the aqueous phase by the addition of 1/10th volume of 3M sodium acetate pH 5.2 and 2 volumes of cold 100% ethanol. DNA precipitation occurred for 15 minutes at -20°C and the DNA pellet recovered by centrifugation at 11600g for 30 minutes at RT.

The pellet was rinsed with 70% ethanol, dried briefly in a heating block (55°C) and resuspended in 30µl of water containing 300µg/ml RNase A (Sigma-Aldrich) and incubated at 37°C for 1 hour. After RNase A treatment the plasmid DNA was further purified by a phenol: chloroform: isoamyl alcohol extraction (25:24:1, v:v:v) and recovered by ethanol precipitation and resuspended in 30µl of water. An aliquot of DNA (3µl) was electrophoresed along with DNA purified from control blue colonies, to identify possible recombinants. Recombinants were identified by their slower migration than non-recombinants in agarose.

Sequencing reactions were carried out by the Australian Genome Research Facility using M13 sequencing primers. Sequence data was compiled using the Multisequence Alignment Program (ANGIS). Sequences were aligned using the 'Pile-up' (ANGIS) program, and aligned sequences were then formatted with the 'Pretty' (ANGIS) program allowing visualisation of the aligned sequences.

4.3 RESULTS

4.3.1 Promoter Methylation status in K562 and K/EPR cells

To test the possibility that required *MDR1* transcription factors may be unable to access their sites *in vivo* due to chromatin condensation caused by CpG methylation, we performed bisulphite mediated genomic sequencing to determine the methylation status of the 66 CpG sites between – 462 to +782bp in the *MDR1* downstream promoter in K562 and K/EPR cells. The sequence region under investigation and the location of the 66 CpG sites are shown in Figure 4.1. Also shown in Figure 4.1 are the locations of the two cycle PCR nested converted strand specific primers, M1/M2 and M3/M4. Twenty individual clones from each of K562 and K/EPR DNA were sequenced.

As shown in Table 4.1, all CpG sites from –462bp to the transcription start site of the *MDR1* gene in K562 cells were unmethylated except for site 17, (–30bp) in one clone. The remaining CpG sites from +4 to +719bp were unmethylated in most K562 clones, except for CpG sites 43 and 44 (+421 and +423bp), that were methylated in all DNA molecules examined from K562 cell DNA. Thus methylation-mediated heterochromatinisation of the promoter cannot account for the absence of expression in K562 cells. Hypermethylation was also not observed in K/EPR clones, although a few individual sites were methylated in a small number of clones. Significantly, CpG sites 11 and 12 located within the –110 GC box, previously suggested to be an NRE, were not methylated in K/EPR cells, thus binding of a repressor is not prevented by methylation in K/EPR cells. Most noticeable however, was the absence of methylation at sites 43 and 44, which were completely methylated in K562 cells.

4.3.2 Protein binding levels at CpG sites 43/44

As the only highly differential site of methylation occurred at CpG sites 43 and 44, oligonucleotides encompassing this region were designed for use in EMSAs (3.2.1) to determine if specific binding of a complex to this site occurred. A radiolabelled binding

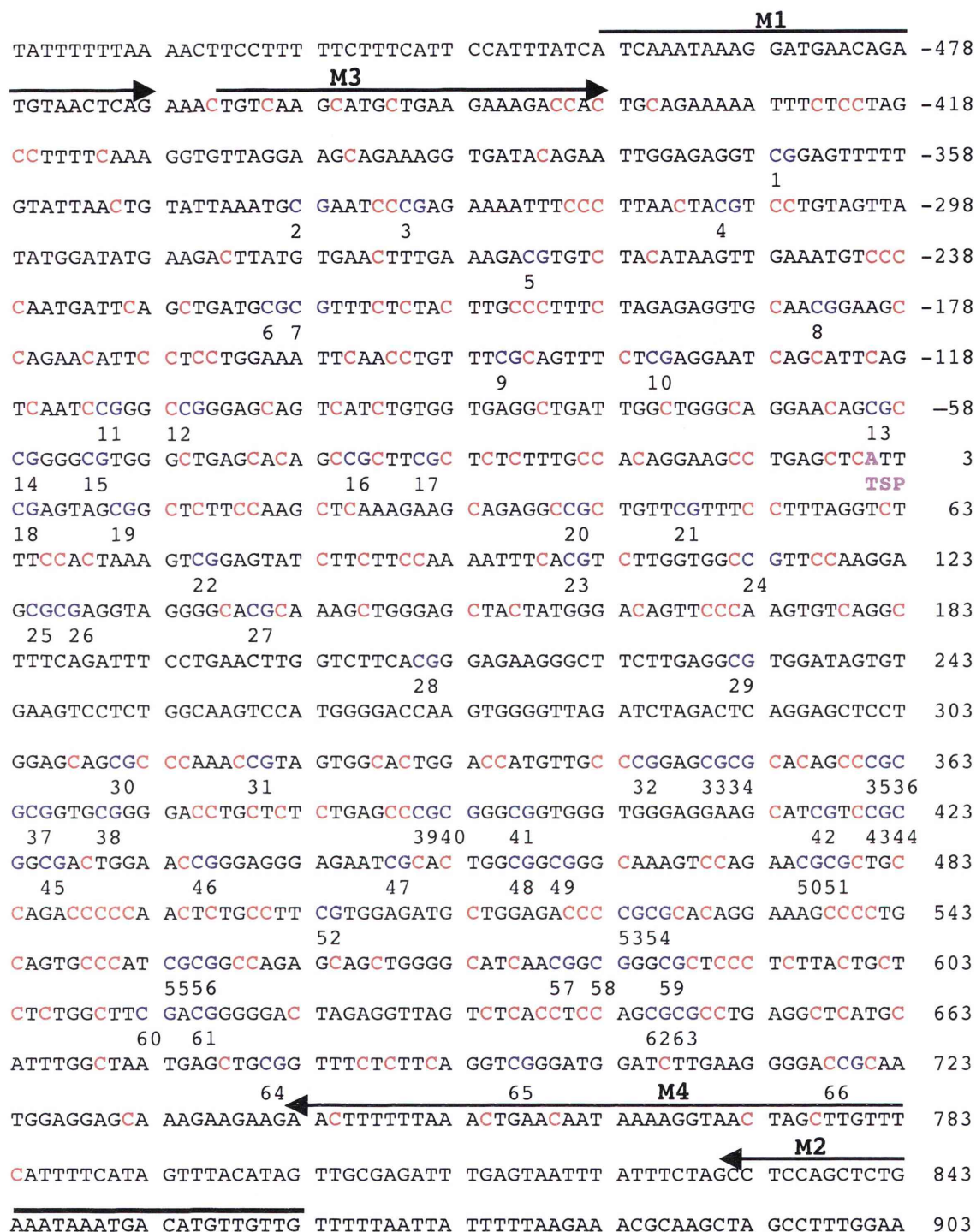


Figure 4.1 Sequence of the *MDR1* promoter. CpG sites are shown in blue and numbered below the sequence. Other cytosines are shown in red. The locations of the converted strand specific primers M1, M2, M3 and M4 are located by arrows above the sequence. The transcription start point is highlighted in pink.

CpG site

[illegible]

Table 4.1 Methylation status of CpG sites in the *MDR1* promoter region (- 462 to +782bp) in K562 and K/EPR cells. Results for 19 and 20 individual clones from K562 and K /EPR cells respectively are shown, where M indicates a methylated CpG site and – indicates an unmethylated site.

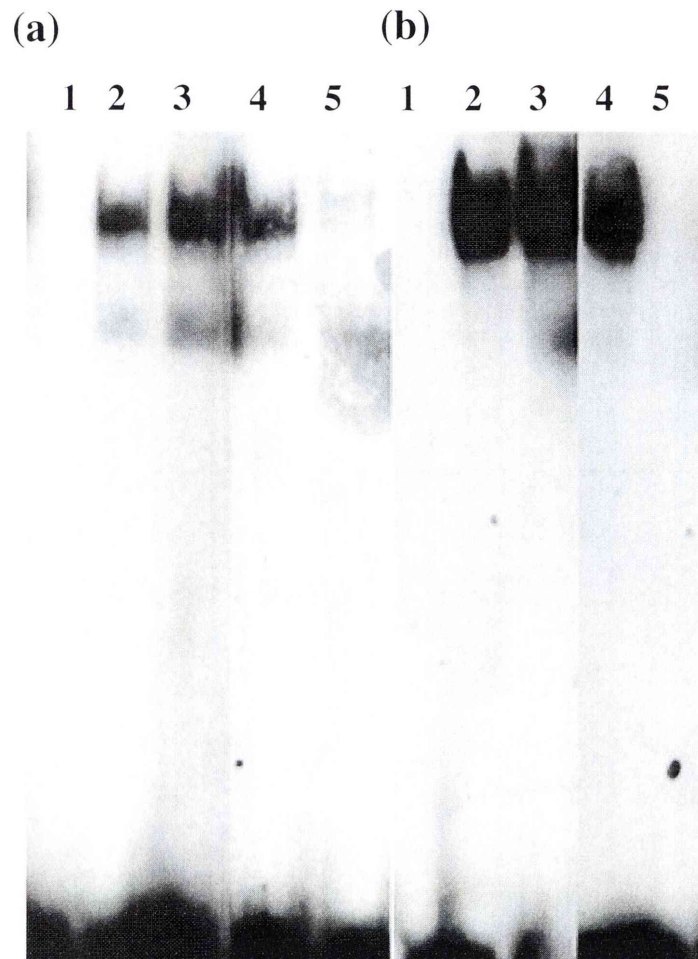


Figure 4.2 EMSA for (a) binding to CpG sites 43 & 44 and (b) USF binding sequence using no extract (lane 1), 3ug of nuclear extract from K562 (lane 2), and K/EPR (lane 3-5). Lane 4 has a 100-fold excess of unrelated competitor DNA and lane 5 had a 100-fold excess of self competitor DNA.

Sp1 LyF-1 43/44 +433
 5' -GGGCGG**TGGGTGG**GAGGAAGCATCGTCCGCGGCGACTGGA
 CCCGCC**ACCCACC**CTCCTTCGTAGCAGGCGCCGCTG**ACCT**-5'
 GT box NF-1

Figure 4.3 Transcription factor analysis of sequence surrounding CpG sites 43 and 44. CpG sites are shown in blue, Sp1 is pink, GT box is red, LyF is highlighted green, and inverted NF-1 sequence is in violet.

Table 4.2 Transcription factor binding sequences.

| Transcription factor | Binding sequence |
|----------------------|------------------|
| Sp1 | GGGCGG |
| GT box | GGGTGG |
| LyF-1 | TGGGAGGAAG |
| NF-1 | TCCA |

revealed that located near the methylation sites is a binding site for Sp1 and a GT box, which is also known to bind members of the SP/XKLF family of transcription factor, of which Sp1 is a member (Bowman and Philipsen, 2002; Lania *et al.*, 1997). Studies have shown that Cytosine methylation in the Sp1 binding sequence does not affect binding of transcription factor Sp1 *in vitro* (Harrington *et al.*, 1988). Moreover the differential methylation sites are not within the Sp1 binding sequence. A binding sequence for the transcription factor LyF-1 is also located near to the differential methylation sites, but again these are not within the LyF-1 binding sequence. LyF-1 is a transcriptional activator of genes but thought to be restricted to genes of B and T-lymphocyte lineages (Lo *et al.*, 1991; Musumoto *et al.*, 2002). NF-1 is a family of transcription factors that have been implicated in the activation and repression of many genes and binds to DNA as dimers to the consensus sequence TTGGC(N)₅GCCAA (Blomquist *et al.*, 1996). Only a partial sequence was found in the sequence surrounding the methylated CpG sites and therefore it is not likely to be of any significance. Thus the significance of the differential methylation at CpG sites 43/44 is as yet unknown.

In view of the absence of any evidence for transcriptional repression of the *MDR1* gene in K562 cells, or for a mechanism mediating transcriptional activation in K562 derived MDR lines, it is possible that the lack of P-gp expression in K562 cells and its upregulation in the K562 derived MDR cell lines may be mediated by post-transcriptional mechanisms, as was recently reported for other K562 derived MDR cell lines (Yagüe *et al.*, 2003).

CHAPTER 5

***MDR1* mRNA Stability Studies**

5.1 INTRODUCTION

The half-life of mRNA molecules may vary from minutes to hours to months. Long lived mRNAs can support multiple rounds of translation, thus producing a larger volume of gene product, whereas short lived mRNAs enable rapid regulatory changes in response to environmental signals. Thus regulation of mRNA stability is another point at which gene expression can be controlled (reviewed in Wilusz *et al.*, 2001). Alteration of mRNA stability can be controlled by variation in the length of the poly-A tail, or by proteins which bind to stability or instability regulating sequences in the mRNA.

Different levels of *MDR1* mRNA were found in the K562, K/EPR and K/EPR drug treated cell lines. To investigate whether an increase in *MDR1* mRNA stability is responsible for the increased levels of *MDR1* mRNA and thus MDR in the K/EPR and K/EPR drug treated cells, the half lives of *MDR1* mRNA were determined following inhibition of transcription by actinomycin D, and compared for each cell line, before and after epirubicin treatment.

5.2 MATERIALS AND METHODS

5.2.1 Determination of Inhibitory Actinomycin D concentrations

To assess mRNA stability, transcription is inhibited by the addition of Actinomycin D and levels of mRNA measured at various times following transcriptional inhibition. To determine the actinomycin D concentration required to inhibit transcription in our cell lines, 1×10^5 cells were plated into 24 well plates in 1mL of media. Cells were incubated for 2 hours with serial dilutions of actinomycin D. Matching solvent only control treatments were also set up to assure inhibition was not due to the DMSO in which the actinomycin was dissolved. After 2 hours the actinomycin D was removed by spinning cells at 500xg for 5minutes and washing in PBS. The cells then were pulsed with $4\mu\text{Ci/ml}$ [^3H]Uridine for 2 hours. After washing in PBS, RNA was isolated from the cells using Tri reagent (Sigma-Aldrich) according to the manufacturers protocol.

Total RNA was resuspended in $25\mu\text{L}$ nuclease free water and $1\mu\text{l}$ was spotted on a microBeta glassfibre filtermat (Wallac, Finland) and dried at 60°C for 50 mins. A meltilex A melt-on scintillator sheet (Wallac, Finland) was then applied to the filtermat by heating at 60°C . Filtermats were then placed in a microbeta sample bag (Wallac, Finland) and quantitated in a microbeta liquid scintillation counter (Wallac, Finland), to measure the levels of RNA synthesised following inhibition of transcription with varying levels of actinomycin D.

5.2.2 *MDR1* mRNA stability determination

Cells were treated for 3 days with epirubicin (David Bull Laboratories, Australia) at a concentration of 5ng/ml for K562 cells and 20ng/ml for K/EPR cells and assayed for paclitaxel cytotoxicity (as described in section 2.2.7 and 2.2.2 respectively).

Exponentially growing cells were then plated into 6 well plates in 5ml of RPMI and treated with $4\mu\text{g/ml}$ actinomycin D. After 0, 3, 10, 20 and 30 hours exposure, RNA was isolated using Tri reagent (Sigma-Aldrich). The concentration of the RNA samples was determined as described in section 2.2.3 and its integrity determined by electrophoresing $10\mu\text{g}$ of total RNA from each sample on 1% Agarose gels with 1x

MOPS (0.04 M Morpholinopropanesulfonic acid (free acid); 0.01 M Na-acetate-3 x H₂O; 1mM EDTA; adjusted to pH 7.2 with NaOH) and 2% formaldehyde running buffer.

5.2.3 *MDR1* RT-PCR

Total RNA was converted into first strand cDNA using a Superscript II kit (Invitrogen). Briefly, 5µg of total RNA was mixed with 75pmoles Oligo(dT) and 0.1mM dNTPs in a total volume of 12µl, heated at 65°C for 5 minutes, then chilled on ice. 0.1 volumes of 10X First strand buffer, DTT to a final concentration of 10mM and 40 units of RNasin (Promega) were added and reactions incubated at 42°C for 5 minutes. 200 units of Superscript II (Invitrogen) was then added and reactions mixed by gentle pipetting, before incubation at 42°C for 50 minutes, followed by a 70°C incubation for 15 minutes to inactivate the enzyme.

The first strand cDNA samples were amplified using a forward primer RT1 (5' CAAAGAAGCAGAGGCCGCTG 3') that is homologous to a sequence in exon I and a reverse primer, RT3 (5'GAAAATACACTGACAGTTGG 3') that is complimentary to a sequence in exon 3. The expected product from these primers is 227bp. As an internal control, β-actin cDNA was amplified in the same reaction as *MDR1* cDNA using the forward primer AF (ATGGATGATGATATCGCCGC) and the reverse primer AR (CGTACATGGCTGGGGTGTG) to produce a 400bp product.

In initial optimisation experiments, primer concentration and cycle numbers were varied to ensure amplification was in the linear phase for both *MDR1* and β-actin. However, final PCR reactions were carried out in 20µl total reaction volumes containing 1x AmpliTaq, buffer (Roche), 1.5mM MgCl₂, 200µM dNTPs, 1µM RT1 and RT3 primers, 0.25µM AF and AR primers, 0.05 units of AmpliTaq DNA polymerase (Roche) and 1µl of cDNA. Reactions were amplified using the following PCR parameters {94°C/2min, 55°C/1min, 72°C/1min} x 1 cycle the {94°C/1min, 55°C/1min, 72°C/1min} x 25 cycles with a final extension of 72°C for 7 minutes. Amplified reactions were run on 2% agarose, 1X TBE gels. Densitometry was performed on gels and the amounts of *MDR1*

cDNA and *β-actin* PCR products were determined by image analysis. The levels in individual samples were normalised by the following calculation,

$$\frac{MDR1 \text{ cDNA intensity}}{\beta\text{-actin cDNA intensity}}$$

To determine the *MDR1* mRNA half life, normalised *MDR1* cDNA levels were expressed as a percentage of the zero time point, and values were plotted against time.

5.3 RESULTS

5.3.1 Optimal Actinomycin D concentration

In order to measure the *MDR1* mRNA half-life following actinomycin D inhibition of transcription, it was first necessary to measure the concentration of actinomycin D required to produce greater than 95% inhibition of RNA transcription in each cell line. This was tested by exposing the cells to serially increasing concentrations of actinomycin D for two hours, then pulsing cells with [³H]uridine and measuring the reduction in [³H]uridine incorporation due to actinomycin D treatment, compared against untreated controls for each cell line. As shown in Figure 5.1, [³H] incorporation into RNA was inhibited greater than 95% by a concentration of 4μg/ml of actinomycin D in both the K562 and K/EPR cell lines.

5.3.2 *MDR1* mRNA half life

K562 and K/EPR cells were treated with epirubicin for 3 days, and, following a short recovery period, assayed for sensitivity to paclitaxel. As was seen previously, K/EPR cells are stimulated to a higher MDR level upon drug exposure, whereas epirubicin treatment had no apparent effect on sensitivity to paclitaxel in K562 cells (Figure 5.2).

The untreated and epirubicin treated K562 and K/EPR cell lines, were then exposed to 4μg/ml of actinomycin for 0, 3, 10, 20 and 30 hours and RNA isolated from the cells at each time point (Figure 5.3a). The levels of *MDR1* mRNA were determined by reverse transcription PCR and normalisation with co-amplified *β-actin* mRNA. In order to

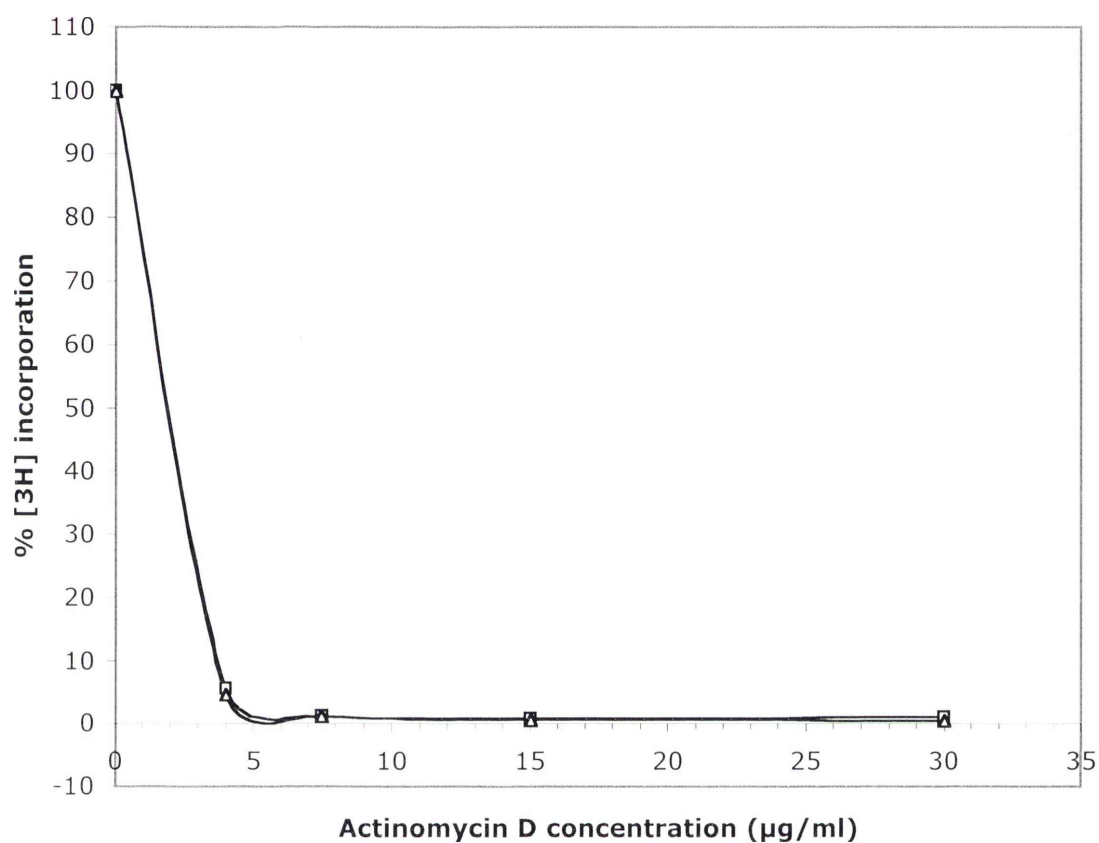


Figure 5.1 Optimal actinomycin D concentration for K562 and K/EPR cells. K562 (□) and K/EPR (Δ) cells were exposed to serially increasing concentrations of actinomycin D for two hours then pulsed with [3H]uridine and the reduction in [3H]uridine incorporation due to actinomycin D treatment was compared against for each cell line and the 95% inhibitory concentration determined.

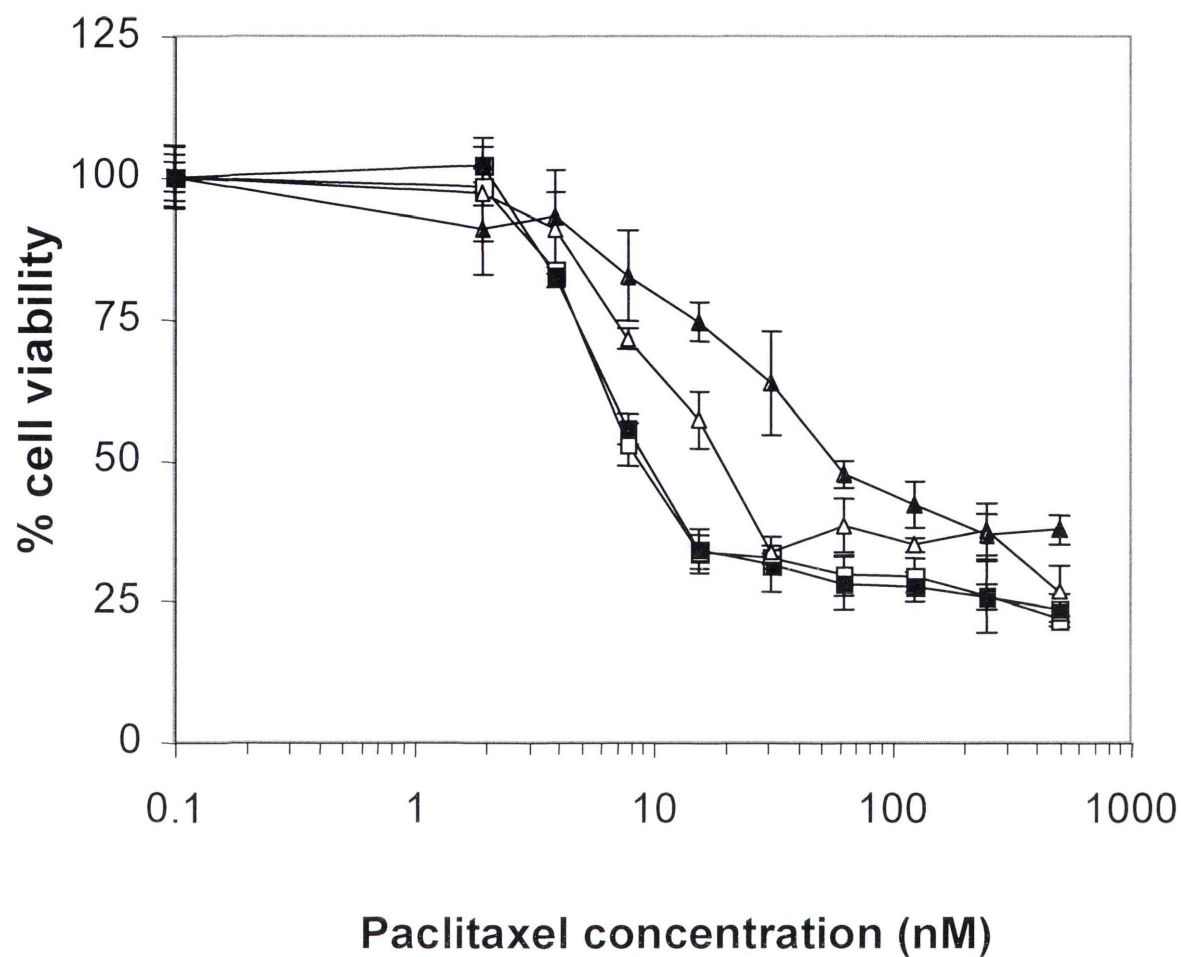


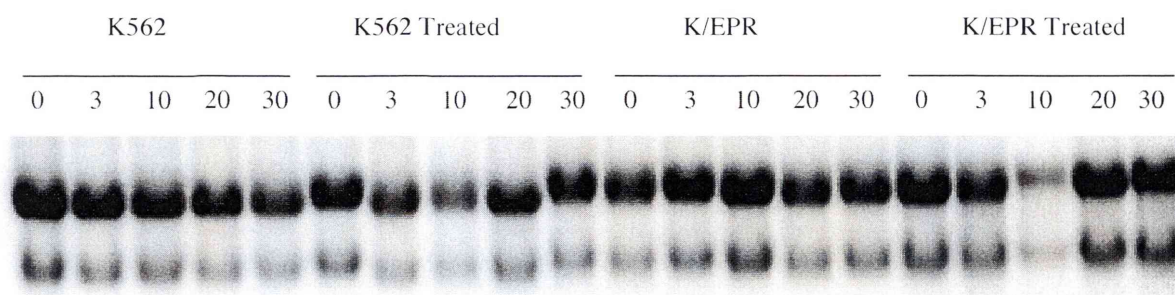
Figure 5.2 The effect of short-term drug exposure on the K562 and K/EPR cell lines. The drug treated K562 (■) and K/EPR cells (▲) were assayed for their resistance to paclitaxel and compared to untreated K562 (□) and K/EPR (△) cells. Points are the mean of triplicate wells and bars are the standard deviation.

ensure both the *MDR1* cDNA and β -actin cDNA were in the linear phase of amplification, several optimisation experiments were carried out whereby the concentration of the primers and the number of PCR cycles were varied. PCRs were performed using 0.25 μ M or 0.5 μ M of β -actin primers and a constant 1 μ M of *MDR1* primers, and all reactions were run at 5, 10, 15, 20, 25, 30, 35x PCR cycles. From this it was determined that 0.25 μ M of the β -actin primers, 1 μ M of the *MDR1* primers in a 25x cycle PCR reaction resulted in linear amplification of both *MDR1* and β -actin cDNA (results not shown).

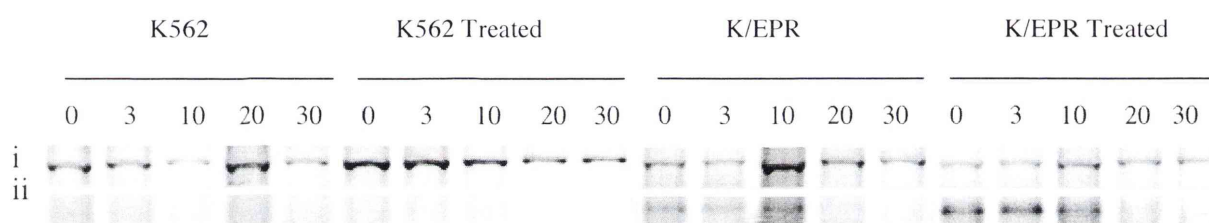
The resulting RT-PCR products for *MDR1* and β -actin can be seen in Figure 5.3b. No *MDR1* RT-PCR product was observed for either untreated or epirubicin treated K562 cells, however *MDR1* RT-PCR product was seen in both the untreated and epirubicin treated K/EPR cells (Figure 5.3b). Determination of the levels of *MDR1* RT-PCR product, normalised with the β -actin internal control reveals higher *MDR1* mRNA levels in epirubicin treated K/EPR cells compared to untreated K/EPR cells, consistent with the previous demonstrations of mRNA levels in Chapter 2. As shown in Table 5.1 the relative amount of *MDR1* mRNA in epirubicin treated versus untreated K/EPR cells was similar to the relative difference in resistance to paclitaxel exhibited by these cells (see Figure 5.2).

However upon graphing the normalised levels of *MDR1* mRNA as a percentage of mRNA remaining against time (Figure 5.3c) no difference in *MDR1* mRNA half life is apparent between the treated and untreated cells, with the half life determined to be approximately 12.5 hours for both untreated and epirubicin treated K/EPR cells. The results of these experiments suggest that an increase in *MDR1* mRNA stability is not the mechanism for induction of a higher level of MDR and *MDR1* mRNA levels after drug exposure in the K/EPR cell line. An increase in mRNA stability could be responsible for the difference between K562 cells and their derived MDR subline K/EPR, however since the levels of *MDR1* mRNA in K562 cells are too low to measure, even by RT-PCR, it was not possible to measure the *MDR1* mRNA half life in K562 cells.

(a)



(b)



(c)

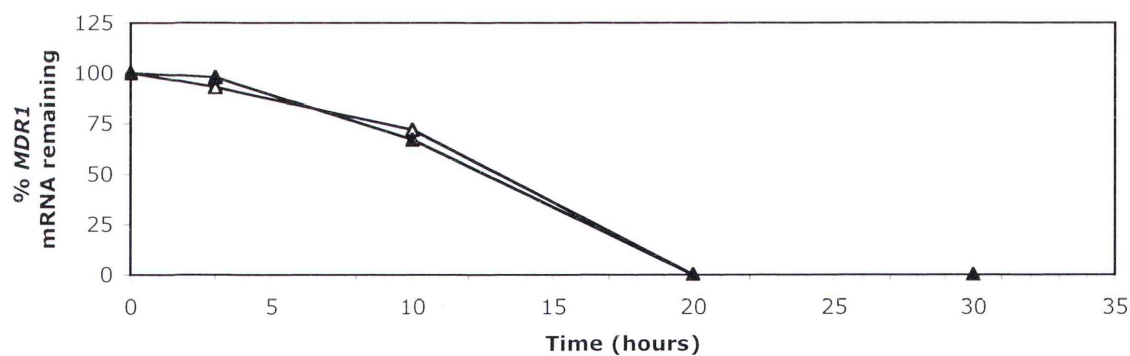


Figure 5.3 *MDR1* mRNA half-life for K/EPR cells before and after Epirubicin treatment. K562 and K/EPR untreated and epirubicin treated cells were exposed to 4 μ g/ml of actinomycin D for 0, 3, 10, 20 and 30 hours. (a) Isolated RNA run on formamide gels to check integrity. (b) RT-PCR products for (i) β actin and (ii) *MDR1*. (c) β -actin normalised percentages of *MDR1* mRNA RT-PCR products remaining after actinomycin D treatment of K/EPR (Δ) and K/EPR epirubicin treated (\blacktriangle) cells.

Table 5.1 Comparison of *MDR1* cDNA levels and paclitaxel resistance before and after 3 days epirubicin treatment.

| | Relative <i>MDR1</i> level¹ | Relative Paclitaxel Resistance ² |
|----------------------------------|---|--|
| Untreated K/EPR cells | 1 | 1 |
| Treated K/EPR cells | 2.6 | 2.9 |

1. *MDR1* mRNA RT-PCR product level relative to that of untreated K/EPR cells (taken from the data shown in Figure 5.3).

2. Resistance levels shown are relative to that of untreated K/EPR cells and refer to the individual IC₅₀ drug concentrations (taken from the data shown in Figure 5.2).

CHAPTER 6

Discussion

6.1 MDR and its reversible induction in K562 MDR sublines

To solve the problem of multidrug resistance in cancer chemotherapy more has to be understood about the underlying mechanisms that cause this phenomenon. This project utilised K562 drug sensitive parental cells and several K562 derived MDR sublines as an *in vitro* model of acquired *MDR1* mediated MDR. The K562 cell line was derived from a patient with chronic myeloid leukaemia and is relatively sensitive to chemotherapeutic drugs (Lozzio & Lozzio, 1979). P-gp expression in K562 cells is generally considered to be negative (Marks *et al.*, 1993; Marks *et al.*, 1996) although some investigators believe that these cells have a low level of expression (Hamada & Tsurdo, 1986, Yague *et al.*, 2003). In the present study no *MDR1* mRNA was detected either by Northern analysis, or by RT-PCR, thus if *MDR1* is expressed it must be very low.

The MDR sublines used were isolated previously by exposure of K562 cells to relatively low levels of drugs such as epirubicin (Marks *et al.*, 1993) and daunorubicin (Hargrave *et al.*, 1995). These MDR sublines express P-gp and the drug resistance is reversed by modulators of P-gp function, indicating that activation of P-gp expression is the cause of the MDR in these cell lines. The levels of drug resistance displayed by the K562 MDR sublines varied, with the relative resistance lowest in the K/EC32 cells, higher in the K/EPR cells and highest in the K/DNR cells. The comparative levels of

resistance correlated with the level of the *MDR1* mRNA in the cells, suggesting that it is controlled by varying *MDR1* transcriptional capabilities in the 3 MDR cell lines. However amplification of *MDR1* gene copy number was ruled out as a possible cause of variation in transcription between the MDR cell lines.

Drug resistance in the K562 MDR cell lines was found to decline over time in drug free culture. However resistance levels could be restimulated in each of the MDR cell lines by a single, short-term, low cytotoxicity, treatment with the original selecting drug or other P-gp substrate drug. Furthermore upregulation of resistance in the MDR sublines induced by short-term drug exposure was accompanied by increased expression of *MDR1* mRNA. These findings suggest that in some cancer cells, expression of P-gp, like that of many proteins with roles in cell detoxification such as liver enzymes, can be induced by toxicant substrates to a higher level when required, and decreased after an extended period of time without drug stimulation.

In a similar study, Hu *et al.* (1995) investigated induction of *MDR1* mRNA by short-term drug exposure in two CEM/A7 derived cells which, like the cell lines in this study, exhibited a low level of drug resistance. One of these cell lines, CEM/A7, had been maintained in the presence of a low level of drug, whereas the other, CEM/A7R, had been grown for two years in the absence of drug. The CEM/A7R cell line was shown by flow cytometric analysis with MRK16 antibody to have decreased P-gp expression compared to the CEM/A7 cells, and this was accompanied by a decrease in drug resistance. Cells from both cell lines were then subjected to short term drug exposure with the anthracyclines epirubicin, daunorubicin or doxorubicin which resulted in an increase in drug resistance in the CEM/A7R cell line but not in the CEM/A7 cell line, which had been maintained in drug.

The decline of MDR levels with time in culture in the absence of drug and re-stimulation upon drug exposure, may also occur in clinical cancer treatment. Patients who at first sight appear not to be expressing P-gp may respond to an initial chemotherapeutic treatment. However chemotherapy failure upon subsequent relapse, may be due to an induction of P-gp expression, and hence MDR, in the cancer cells

caused by the first drug treatment, thereby enabling the cancer cells to resist cytotoxicity from subsequent drug treatments (Hu, *et al.*, 1995; Nooter and Herweijer, 1991).

In this study, only P-gp substrates were able to induce *MDR1* expression, with no induction seen with the non-Pgp substrate drug cisplatin. A possibility for the apparent failure of MDR induction by cisplatin could be the reported sensitisation to paclitaxel of cisplatin resistant cells. Yamamoto *et al.*, (2000) demonstrated that KFr13, a cisplatin resistant human ovarian carcinoma cell line, acquired increased sensitivity to paclitaxel, compared with its cisplatin sensitive parental Kfr28 cell line, thus suggesting that cisplatin sensitises the Kfr13 cells to paclitaxel. Therefore it is possible that in K562 MDR cell lines an induction of MDR could be masked in cisplatin treated cells by using paclitaxel to assay any changes in cell resistance. However vinblastine cytotoxicity assays performed on cisplatin treated cells also indicated no induction of drug resistance, confirming that cisplatin did not induce *MDR1* activity in our model.

In contrast to the inducibility of drug resistance in the K562 MDR sublines, treatment of the parental K562 cells with a similarly low cytotoxicity dose of epirubicin, had no effect on sensitivity to the *MDR1* substrate paclitaxel. Thus the absence of *MDR1* mRNA, and inability to be induced by short term drug exposure, indicates a fundamental difference between the K562 cells and its derived MDR sublines. This would mimic the situation of a cancer that responded to multiple chemotherapy rounds, perhaps only failing after a further mutation led to the activation of *MDR1* expression and development of an MDR cancer. This is an apparent contradiction to a previous study by Chaudary and Roninson (1993) which indicated that transient treatment with the non P-gp substrate drug cisplatin, induced MDR levels and increased *MDR1* mRNA in K562 cells. However in the latter study, K562 cells were treated with a much higher and more cytotoxic dose (2 days with 3 μ g/ml cisplatin) than we used here on K562 derived K/EPR MDR cells (16 hours with 0.5 μ g/ml). De Jongh *et al.*, (2001) demonstrated an average peak plasma concentration of cisplatin less than 0.7 μ g/ml in patients receiving 70mg/m² of cisplatin. Similarly, Gelderbloom *et al.*, (2001) found a maximum unbound cisplatin concentration of 0.81 μ g/ml in patients treated for three

hours with 70mg/m² cisplatin. Thus it is possible that the increase of MDR after high level cisplatin treatment seen by Chaudary and Roninson (1993) could have resulted from a mutational change in the K562 cells resulting in production of an MDR cell line. Alternatively it may result from a general stress response mechanism that stabilises *MDR1* mRNA, rather than as a result of increased *MDR1* transcription. It is therefore possible that highly cytotoxic cisplatin treatment could activate *MDR1* expression in the normally non-expressing K562 cells, as well as K562 derived MDR cell lines.

Recent studies have demonstrated that rifampicin, which induces intestinal activity of cytochrome P450, can also induce *MDR1*. Cytochrome P450 is mainly found in human liver, the main organ involved in drug and toxin removal, but a large amount is also found in the small intestine and it is also present in pancreas, brain, lung, adrenal gland, kidney, bone marrow, mast cells, skin, ovary and testis (Chang and Kam, 1999). Using immunohistochemical staining and Western blots, Greiner *et al.* (1999) demonstrated induction of intestinal P-gp *in vivo* by taking duodenal biopsies in 8 healthy people before and after they were administered rifampicin. Westphal *et al.* (2000), using RT-PCR, showed increased *MDR1* mRNA expression in 6 out of 8 duodenal biopsies from healthy volunteers after administration of rifampicin, although all 8 of the biopsy samples showed increased P-gp protein levels in both immunohistochemistry and Western blot assays.

In a similar study Geick *et al.* (2001) demonstrated rifampicin induction of *MDR1* in a time and concentration dependant manor in LS174T cells, a human intestinal cell model. An SXR nuclear response element located -7852 to -7837bp upstream of the *MDR1* promoter was identified as essential to *MDR1* induction by rifampicin (Geick *et al.*, 2001). However in the current study rifampicin had no effect on resistance in the K/EPR cell line, thus it is possible that induction of P-gp by rifampicin is selective to the intestines or gut wall or to tissues of high cytochrome P450 expression.

6.2 Transcriptional regulators of MDR1

The most immediately obvious candidate for mediating reversible induction of *MDR1* mRNA upon exposure to P-gp substrates is transcriptional activation. Numerous

studies have indicated that an inverted CCAAT box (Y-box) located in the sequence between –93 and –68 upstream of the transcription initiation site has an important role in *MDR1* gene transcription (Hu *et al.*, 2000; Jin and Scotto, 1998; Ohga *et al.*, 1998; Ohga *et al.*, 1996; Sundseth *et al.*, 1997; Goldsmith *et al.*, 1993; Asakuno *et al.*, 1994) however there are conflicting reports regarding the identification of the Y-box binding factor involved in regulation.

Studies of the KB cell line stably transfected with *MDR1*:CAT reporter constructs demonstrated that exposure to UV or cytotoxic drugs caused an increase in *MDR1* promoter activity (Asakuno *et al.*, 1994; Uchiumi *et al.*, 1993). Exposure to cytotoxic agents such as cisplatin and UV light also caused an increase in YB-1 mRNA levels (Ohga *et al.*, 1996). In 1998 Ohga and colleagues demonstrated that the increases in *MDR1* promoter activity resulting from exposure of the *MDR1*:CAT transfected KB cells to cytotoxic drugs or UV irradiation was decreased by transfection with a YB-1 antisense expression plasmid. However no effect on promoter activity was seen on transfection with antisense YB-1 in KB cells that had the Y-box deleted from the *MDR1* promoter (Ohga *et al.*, 1998) suggesting that interaction of YB-1 with the Y-box in KB cells is important for genotoxic stress induction of *MDR1*. A previous report that deletion of the Y-box from an *MDR1* promoter construct had no effect on promoter activity on KB cells Cornwell (1993), indicated that the Y-box sequence is not essential for normal *MDR1* transcription in these cell lines.

However, in contrast to these results, other reports have indicated that the Y-box is essential for normal transcription and that the factor, NF-Y, binds to the Y-box in the *MDR1* promoter (Hu *et al.*, 2000; Jin & Scotto, 1998; Sundseth *et al.*, 1997). For example Sundseth *et al.*, (1997) reported that in tumour cell lines HCT116, HepG2, KB3-1 and Saos2, NF-Y, and not YB-1 binds to the Y-box elements on the *MDR1* promoter. It was also shown that a double point mutation in the Y-box sequence between –89 and –70 reduced activity of the promoter by 5-10 fold. Thus in these cell lines the Y-box appeared to be essential for maintaining normal *MDR1* activity. Similar results were found in a study on SW620 colon carcinoma cells and 2780 ovarian carcinoma cells, where deletion or mutation of the sequence between –89 and –70

resulted in a decrease in promoter activity of 5-10 fold (Goldsmith *et al.*, 1993). Thus the role of the Y-box in *MDR1* regulation, though clearly important, appears unclear. In some cells it appears to be constitutively essential to *MDR1* activity and in others to mediate upregulation of *MDR1* in response to genotoxic stress. It was therefore speculated by both sides that the Y-box binding factor interacting with the *MDR1* Y-box may differ in different cell lines and that inherent differences in the cell lines may be the reason (Ohga *et al.*, 1998; Sundseth *et al.*, 1997). For example the HCT116, HepG2, and Saos2 tumour cells used by Sundseth *et al.*, (1997) have inherently high *MDR1* expression and the presence of the Y-Box was found to be essential in these cells for basal transcription of *MDR1*. KB cells however are induced to a higher level of *MDR1* expression as a result of genotoxic stress on the cells and thus the Y-box in KB cells may be important for stress induction and not basal transcription of *MDR1*.

However a more recent study by Hu *et al.*, (2000) has challenged this by re-investigating the Y-box binding factor involved in activation of *MDR1* by UV irradiation in KB-3-1 cells. As with the previous studies Hu *et al.*, (2000) found that the CCAAT box was required for activation of *MDR1* transcription by UV light. However unlike the previous work, an anti-NF-Y antibody and an anti-YB-1 antibody was used in EMSAs to identify the Y-box binding protein interacting with a double stranded Y-box probe. Results from this indicated that NF-Y bound to the Y-box and not YB-1. In the present study it was also demonstrated by gel supershifting with anti-NF-YA antibody that the factor binding to the Y-box in K562 derived MDR cells is NF-Y.

YB-1 has been shown to preferentially bind to single stranded DNA (Hasegawa *et al.*, 1991; Kolluri *et al.*, 1992; Tafuri and Wolffe, 1992; MacDonald *et al.*, 1995; Bayarsaihan *et al.*, 1996; Mertens *et al.*, 1997; Ohmori *et al.*, 1996; Kloks *et al.*, 2002). However EMSAs using a single stranded YB-1 binding sequence showed no specific binding of nuclear factors from our K562 or K/EPR MDR subline to the single stranded oligonucleotide. This gives further support for the identification of NF-Y, and not YB-1 binding to Y-box in the *MDR1* promoter of K562 derived cell lines. However, the induction of MDR levels and *MDR1* mRNA in the K562 derived MDR cell lines upon

drug treatment does not correlate with increased nuclear NF-Y levels in these cells as measured by EMSAs performed on protein extracts isolated from drug treated cell lines.

It has been recently shown that *MDR1* induction in some cell lines can be inhibited by treatment with the novel anti-cancer drug Ecteinascidin-743 (ET-743), derived from the murine tunicate *Ecteinascidia turinata* (for review see Scotto 2002). Whilst *MDR1* activation is inhibited by ET-743, basal transcription levels of the gene appear to remain relatively unaffected (Jin *et al.*, 2000). A study by Bonfanti *et al.* (1999) examined the affect of ET-743 on the binding of various transcription factors to their DNA consensus sequences by pre-incubating the factors of interest with ET-743 and then looking for *in vitro* binding inhibition by EMSA with the DNA consensus sequences. Both Sp1 and NF-Y were included in this investigation. Of the transcription factors assessed, the binding of TATA binding protein (TBP), E2F, and SRF were found to be inhibited by 50 - 300 μ M ET-743. More interestingly, NF-Y was inhibited by ET-743 at a concentration as low as 10-30 μ M ET-743. Thus these results suggest NF-Y binding to the Y-box is a target for ET-743 inactivation of gene induction.

Sp1 binding however was found not to be affected by ET-743. Jin *et al.*, (2000) demonstrated that SW620 colon carcinoma cells transfected with an *MDR1*/luciferase reporter construct and exposed to either TSA, sodium butyrate or UV irradiation showed increased *MDR1* promoter activity. However, this increased *MDR1* promoter activity was abolished in the presence of 50nM ET-743, which is a physiologically relevant concentration. This outcome was also reflected in nuclease protection assays on the RNA from SW620 cells exposed to *MDR1* inducing agents for 24 hours with, and without the presence of ET-743 (Jin *et al.*, 2000). Induction of *MDR1* expression seen after treatment with TSA or sodium butyrate was abrogated in the presence of ET-743. Previously, *MDR1* induction by these agents in this cell line have been shown to be mediated through interaction of NF-Y and the -93 and -68 *MDR1* promoter Y-box (Jin and Scotto, 1998). However EMSAs using nuclear extract from untreated and ET-743 treated SW620 cells showed no difference in NF-Y levels (Jin *et al.*, 2000). A similar study also concluded levels of NF-Y binding to the *HSP70* gene promoter, which is also an inducible promoter inhibited by ET-743 and has two NF-Y activated Y-boxes, was

unchanged with ET-743 treatment (Minuzzo *et al.*, 2000). These studies, together with the current study demonstrate that, whilst NF-Y binding to the Y-box may be required for induction, changes in NF-Y levels per se are not responsible for *MDR1* drug induction. This however does not go to say that NF-Y plays no role in *MDR1* up-regulation, it is possible that up-regulation could be mediated by changes occurring to factors acting either directly or indirectly on NF-Y.

6.3 GC box binding protein involvement in *MDR1* activation

As for NF-Y, the levels of the factor binding to the –55GC box also did not differ between K562 and the K/EPR MDR cell line and did not change after drug exposure. The –55GC box is one of several GC boxes found within the *MDR1* promoter, however it appears to be the most essential in terms of basal transcription (Cornwell and Smith, 1999a, Sundseth *et al.*, 1997). Evidence for this comes from mutation and deletion experiments on the region containing the –55 GC box. In the case of both deletion and mutation a significant reduction in promoter activity was observed, and in one instance a point mutation in the –55 GC box gave a 60-70% decrease in promoter activity (Sundseth *et al.*, 1997). Sp1 is an activator of many promoters and is believed to behave as an activator of *MDR1* transcription through binding at the –55 GC box. This has been demonstrated by co-transfecting an *MDR1*-luciferase reporter gene with either an Sp1 cDNA construct or vector control into cells that do not express Sp1. Results from this experiment showed that the *MDR1* promoter was activated 15 fold higher in the presence of Sp1 compared to the vector only control construct (Cornwell and Smith, 1999a).

High levels of expression of the –55 GC box binding factor Sp1 have been demonstrated in some human cancers, for example Kitadai *et al.*, (1992) demonstrated Sp1 mRNA expression in 12 out of 18 tumours at levels higher than normal mucosa. Likewise, Lietard *et al.*, (1997) found increased Sp1 levels in human hepatocellular carcinomas. Although increased levels of Sp1 have not been seen in the K562 MDR cell lines in this study, the absolute levels of Sp1 in the cells may not be critical to regulation of *MDR1* transcription. For example the –55 GC box in the *MDR1* promoter contains overlapping

Sp1 and Egr1 motifs. Egr1 is a member of a family of transcription factors known as early growth response (Egr) factors. Recombinant Egr1 has been shown to bind the –55 GC box region (Cornwell and Smith, 1999a) and participates in *MDR1* regulation in some phorbol ester sensitive cell lines (McCoy *et al.*, 1995). Overlapping Sp1 and Egr-1 motifs are found in a number of genes. In the majority of genes containing overlapping Sp1/Egr 1 sites, Egr-1 binds with higher affinity than Sp1, thus when present, inhibits Sp1 binding (Cui *et al.*, 1996; Ebert and Wong 1995; Khachigian *et al.*, 1995). The exact interaction of Egr-1 and Sp1 with the *MDR1* –55 GC box is not yet understood (Cornwell and Smith, 1993a). Sp3 is another member of the same family of transcription factors as Sp1 and is able to bind to the same recognition sequence as Sp1. Unlike Sp1, which is predominantly a transcriptional activator, Sp3 can act as either a repressor or an activator (for review see Philipsen and Suske, 1999). In many genes Sp3 can compete for binding with Sp1 and thus repressing Sp1 mediated transcriptional activation of that gene. Therefore it is possible that in the case of *MDR1*, where Sp1 is essential for basal transcription, the relative abundance of the Sp1 and Sp3 may be involved in transcriptional regulation.

6.4 NRE binding protein involvement in *MDR1* activation

Negative regulation of the *MDR1* promoter via an NRE containing a CAAT-like motif at –118 to –113 was identified in an MCF-7 breast cancer cell line where deletion of the CAAT motif resulted in a 12-fold activation of *MDR1* in MCF7 cells, but had little effect in an MDR MCF-7/Adr cell line (Ogretmen and Safa, 1999). Consistent with the absence of repression in the MCF-7/Adr cells the NRE binding protein, identified as a hybrid NF- κ B/p65-cFos factor, was detected by EMSA analyses in MCF-7 cells but not in MCF-7/Adr cells. However results from the current study indicate that this is not the case in K562 derived cell lines as the protein complex binding to this NRE is present at similar levels in both K562 and K/EPR cells.

The finding that nuclear levels of NF- κ B, SP-1 and NRE-binding complex are present at equivalent levels in drug sensitive K562 cells as in derived MDR sublines indicates that

changes in levels of these factors alone is not responsible for up-regulation of *MDR1* transcription and P-gp. However, as stated previously in section 3.3.5, the binding or transactivation ability of transcription factors *in vivo* may be influenced by protein-protein interactions or specific modification of binding factors. Hence, reporter gene studies were carried out.

6.5 *MDR1* transfection studies

6.5.1 *MDR1* promoter

In transfection studies, the -88/+76 *MDR1* promoter reporter, which contains the Y-box and the GC box, was equally effective in driving reporter gene expression in K562 cells as it is in K/EPR cells. This indicates that differential transcription factor interactions at either of these two sites are not important in determining the differential expression of P-gp in these cell lines. Moreover differential transcription factor interactions at either of these two sites were not increased upon drug treatment, despite increase in *MDR1* mRNA in K/EPR cells by drug treatment.

6.5.2 Downstream region

Studies from this laboratory have indicated that the downstream region of the *MDR1* promoter may contain negative regulatory elements in the +283/+392 and +329/+455 regions. Although the 2 constructs have a 63bp overlap, to confirm the exact location of this repressor element, further transfection studies using need to be performed to determine whether the regulatory element is present the region common to both constructs, or if there is more than one negative regulatory element within the downstream region. Previous *in vitro* footprinting studies have the presence of 3 footprints within the +283/+455 region of the *MDR1* promoter (Desiderato, PhD thesis, 1999). Although it is not known what proteins bind to these footprinted regions, Sigscan analysis of the sequence indicates that several transcription factors have consensus sequences within the footprinted regions and that many of these have been shown to be involved in negative regulation of genes. Examples of these transcription factors include LBP-1 (Parada *et al.*, 1995), AP-2 (Jiang *et al.*, 2000), NF-1 (Li *et al.*, 1997), Sp1 and Sp1 family members (GKLF, TIEG2, BKLF, EKLF, AP-2rep) (Lania *et al.*, 1997;

Philipsen and Suske, 1999) and CF-1 (Zwicker *et al.*, 1997). When K/EPR cells were transfected with the constructs containing the +283/+392 or +329/+455 regions, promoter activity was seen to be clearly repressed compared to cells transfected with the promoter construct alone.

6.6 Effect of TSA on MDR levels

Differences in the levels of histone acetylation and chromatin structure in sensitive and drug resistant cell lines may result in increased or reduced DNA accessibility to important *MDR1* promoter transcription factors and hence levels of *MDR1* transcription. As previously outlined in section 1.3, the balance of histone acetyltransferases and deacetylases can determine the resulting amount of histone acetylation, and thus whether a gene is active or transcriptionally silent (Wolffe, 1996). Originally isolated as an antifungal antibiotic from *Streptomyces hygroscopicus* (Tsuji *et al.*, 1976), TSA has been shown to induce cell differentiation, cell cycle arrest and reversal of transformed cell morphology (Yoshida *et al.*, 1995). TSA is a known specific inhibitor of histone deacetylase (Yoshidai *et al.*, 2001).

In the previous study by Jin and Scotto (1998), SW620 human colon carcinoma cells were treated with 300nM TSA. RNase protection assays using RNA extracted from these cells at various time points showed that increased *MDR1* mRNA levels were observed after 6 hours of TSA exposure. This study also demonstrated in SW620 cells stably transfected with an *MDR1*/luciferase reporter construct (containing sequence from -1202 to +118 of the *MDR1* promoter) promoter activity increased 10 to 14 fold after the cells were exposed to TSA. Mutation studies of *MDR1* promoter sequences in SW620 cells transiently transfected with *MDR1*/luciferase constructs went on to indicate that in this cell line, an intact Y-box and the transcription factor NF-Y was essential for the induction response of the *MDR1* promoter to TSA (Jin and Scotto, 1998), indicating that in those cell lines, histone acetylation levels around the Y-box are controlling features.

In this study, a 16 hour exposure of K562 cells to 50nM or 200nM TSA resulted in a two fold increase in the epirubicin drug resistance of these cells. The effect of TSA exposure to K562 cells suggest that, in the usually sensitive K562 cells, TSA exposure created an alteration in the ratio of histone acetyltransferase and histone deacetyltransferase, whereby inhibition of histone deacetylases lead to increased epirubicin resistance possibly due to increased acetylation and subsequent activation of *MDR1* in these cells. In contrast to this, there was no associated increase of epirubicin resistance after 16 hour TSA treatment of the MDR K/EPR cell line, possibly due to the cells already having a higher level of acetylation than the K562 cells.

6.7 Methylation status of the *MDR1* promoter

DNA methylation of key promoter sites has been implicated in transcriptional regulation of many genes (Bird, 1992). Bisulphite genomic sequencing of K/EPR cell DNA showed that this cell line was completely unmethylated at all CpG sites in the promoter region from – 462 to +782bp. The corresponding results for K562 cell DNA showed there were two distant downstream sites methylated. The presence of this small amount of methylation is unlikely to cause wholesale promoter heterochromatinisation. EMSA studies on this region showed a DNA protein complex binds to this region in both cell lines was present in both cell lines. It is possible that this protein may not be able to bind *in vivo* in the K562 cells due to the methylation in this region. To ascertain this, *in vivo* foot printing could be performed on both cell lines.

In a previous study it was reported that CpG sites at –111 and –106, close to the CAAT containing NRE (–118 to –113) and to a further possible NRE reported in KB-8-5 cells at –110 to –103 (Cornwell and Smith 1993) were methylated in MDR K562/ADM cells and unmethylated in the drug sensitive parental K562 cells (Ando *et al.*, 2000). The authors suggested that activation of *MDR1* in the K562/ADM cells was due to methylation of these sites and hence decrease in accessibility to the –110 repressor binding site. However, in the latter study, only 4 of 20 K562/ADM clones sequenced were found to have this methylation, and these clones were reported to be methylated at

all 28 CpG sites tested (from -389 to +231bp) and thus will undoubtedly not express *MDR1* at all, due to chromatin condensation and inhibition of transcription factor binding. It appears likely that those particular four clones were revertants to methylated and *MDR1* silenced, drug sensitive clones. Cell lines in culture appear able to become methylated (or unmethylated according to circumstance) relatively readily and care must be taken to ensure they continue exhibiting the selected phenotype.

Other studies have reported partial methylation of site 8 (-184bp) in K562 DNA and partial methylation of sites 1 (-367bp) and 3-6 (-331 to -220) in a K562/ADM cell line, however the number of clones on which this was based was not stated (Efferth *et al.*, 2001). Partial methylation at sites 9 (-145) 17 (-30) and heavier methylation at downstream sites from 28 (+211) to 41 (+397) were also reported in 4 to 7 clones from K562 cells (Frxyell *et al* 1999). As stated above methylation can vary with cell line culture history and so patterns will not necessarily be identical in different laboratories, however all the data reported, including the more extensive data reported here, indicates there is no evidence for local, methylation directed heterochromatinisation and silencing of the *MDR1* gene in K562 cells.

6.8 Stability of *MDR1* mRNA in K562 MDR sublines

It was recently reported that for some K562 derived MDR cell lines, different from those used in the current study, P-gp regulation was mediated by post-transcriptional mechanisms, namely mRNA stability (Yagüe *et al.*, 2003). Yagüe *et al.*, (2003) reported that short term exposure of K562 cells to cytotoxic drugs resulted in increased levels of *MDR1* mRNA in the treated cells. Using actinomycin D to inhibit transcription, these authors reported that this increase in *MDR1* mRNA in K562 cells was due to increased mRNA stability and not transcriptional activation. In contrast, in our studies *MDR1* mRNA was undetectable in K562 cells, both before and after drug treatment. It is possible that in K562 drug treated cells the levels although increased were still too low to measure by RT-PCR. However, drug treatment of the K562 cells also resulted in no increase in MDR levels and thus, even if *MDR1* mRNA was stabilised, it did not result

in significant changes in P-gp expression. Such an occurrence would be consistent with the results reported by Yagüe *et al.*, (2003) since they could not demonstrate any increase in P-gp expression in K562 drug treated cells.

The Yagüe *et al.*, (2003) study differs from the current study specifically in respect to the levels of drugs used in the induction of MDR in K562 cells. To test drug induction of *MDR1* in K562 cells Yagüe *et al.*, (2003) exposed K562 cells for 3 days to various cytotoxic drugs, including vinblastine. The concentrations used for these inductions resulted in greater than 50% macroscopic change in cell morphology. For example K562 cells were exposed to 22uM vinblastine for 3 days, which is approximately 10,000 fold higher than the vinblastine IC₅₀ determined in this report (section 2.3.1). Although we used epirubicin and not vinblastine to treat K562 cells, the concentration of epirubicin used was equivalent to an IC₂₅ for a 3 day assay, which is significantly less cytotoxic than the treatment used by Yagüe *et al.*, (2003). It is important to note that the increase in *MDR1* mRNA seen by Yagüe *et al.*, was not accompanied by an increase in functional P-gp in these cells, as determined by flow cytometry using UIC2 and C219 P-gp specific antibodies.

In this study, epirubicin treatment of K/EPR cells did however increase both MDR and *MDR1* mRNA levels in these cells however the rate of the *MDR1* mRNA decay was the same in both the untreated and drug treated cells lines, with the *MDR1* mRNA half life determined to be 12.5 hours. This half life is comparable to the 15-20 hour *MDR1* half life in K562 drug resistant cell lines reported by Yagüe *et al.*, (2003). Thus, in contrary to Yagüe *et al.*, (2000), we did not find increased mRNA stability in K562 MDR cells after short term cytotoxic exposure.

The K562 MDR sublines used by Yagüe *et al.*, (2003) were developed by a one step selection with cytotoxic drugs that resulted in 99.9% cell death after 14 days in culture. The K/EPR cell line used here was developed through several intermittent exposures over a 2 month period of K562 cells to 20ng/ml (34nM) of epirubicin each for 3 days, a concentration which resulted in less than 50% inhibition of cell growth, (Hargrave *et*

al., 1995). Unfortunately the Yagiie *et al.*, (2003) paper did not look at induction of already drug resistant cell lines with further drug exposure.

The high levels of cytotoxic drugs used by Yagiie may account for the reported increased mRNA stability rather than an actual increase in transcription. As discussed previously several studies have demonstrated that the levels of the transcription factor YB-1 is increased in stress induced cells. Ohga *et al.*, (1996) showed that YB-1 mRNA levels increase following genotoxic stress with agents such as cisplatin and UV light. All of these studies used relatively high levels of cytotoxic stress on the cells. It is possible that high levels of stress increase levels of YB-1. Stein *et al.*, (2001) found that when cells were stressed with hyperthermia, YB-1 was translocated to the nucleus and this corresponded with an increase in *MDR1* and *MRP1* levels. Stein *et al.*, (2001) also found that while YB-1 stably transfected HBL-100 cells showed increased *MDR1* promoter driven CAT expression, an increase was also seen in *MRP1* driven CAT expression and the *MRP1* promoter contains no Y-box motif. It is likely that the YB-1 had a post-transcriptional involvement in *MDR1* up regulation. YB-1 is a multifactorial protein that may be involved in transcriptional activation and repression, mRNA binding, mRNA stabilisation and translational control (Copowski *et al.*, 2001; Didier *et al.*, 1988; Steinia *et al.*, 2000; Ansari *et al.*, 1999; Bouvett *et al.*, 1995; Ranjan *et al.*, 1993; Chen *et al.*, 2000). However, it is thought that YB-1 is more likely to be an RNA binding-protein than a double stranded DNA binding protein (Chen *et al.*, 2000). Therefore exposing cells to high levels of some cytotoxic drugs may increase the levels of YB-1 which in turn binds to and stabilises many different mRNA transcripts.

6.9 Future directions

There are still many unanswered questions regarding the regulation of the *MDR1* gene in the K562 MDR sublines, and indeed other cell lines and cancers *in vivo*. Discrimination between transcriptional and post-transcriptional mechanism can be ascertained by performing nuclear run-on experiments, which allow the direct measurement of the rate of transcription of a specific gene. These assays work by

radiolabelling newly synthesised RNA in isolated nuclei (Greenberg, 1987). These experiments were previously attempted in the K562 and K562 MDR cells lines, however lack of sensitivity failed to produce a reliable result and thus were not included. However if the sensitivity of the run-on experiments could be improved then these experiments could provide an invaluable insight into the mechanism of *MDR1* regulation in these cell lines.

The binding levels of various transcription factors thought to be involved in the regulation of the *MDR1* promoter were assessed in the K562 and MDR sublines by EMSAs. Although no variation was found between drug sensitive and drug resistant cell lines, EMSAs only indicate if these binding factors are present in the cell and do not actually reflect whether the factors are actually binding *in vivo*. To determine what intracellular DNA–protein interactions are actually occurring in whole cells *in vivo* footprinting could be carried out. *In vivo* footprinting not only indicates the study of which proteins are binding, but also can allow some indication of how protein- DNA conformation changes, which all give insight into the gene regulation mechanism of the cell (Zaret, 1997). This technique could also reveal the position of nucleosomes and thus the chromatin structure of the *MDR1* promoter. To determine if methylation is directly effecting protein binding to the two CpG sites 43 and 44, that are methylated only in the K562 cells and not the K/EPR MDR cells, EMSAs using methylated oligonucleotides could be performed.

RT-PCR of K562 cDNA failed to detect *MDR1* mRNA and therefore an *MDR1* half life could not be determined for K562. Real time PCR has far greater sensitivity than RT-PCR and may be used to assess mRNA decay rate in K562 and compare it to the stability of *MDR1* mRNA in drug resistant cell lines.

6.10 Conclusions

The K562 MDR model provided an interesting model for the examination of mechanisms that may be a factor in the activation of the *MDR1* gene in cancer cells. It was shown that the K562 derived drug resistance cell lines showed a classical MDR phenotype that is due to the expression of the *MDR1* gene which gives rise to the multidrug transporter P-gp. Secondly it was shown that the level of MDR of the cells correlated with the level of *MDR1* mRNA expressed by the cells. However activation of *MDR1* expression and differences in levels of *MDR1* mRNA between drug resistant cell lines was found not to be due to the amplification of *MDR1* gene copy number. It was also demonstrated that the level of MDR and the expression of *MDR1* in these lines could be induced with exposure to P-gp substrate drugs such as epirubicin, daunorubicin and paclitaxel, but not non P-gp substrates such as cisplatin or rifampicin. Epirubicin and paclitaxel induced the MDR of the cells in as little as 4 hours, suggesting that transcriptional activation of the *MDR1* gene is likely to be occurring after drug treatment of the cells. Short term drug treatment with P-gp substrate drugs was not able to induce levels of MDR in the parental K562 cells. However, exposure of K562 cells to 50nM or 200nM TSA increased epirubicin resistance 2-fold in these cells but not to K/EPR cells, possibly due to increased DNA acetylation and subsequent activation of *MDR1* in the K562 cells.

The binding of transcription factors to the consensus sequences present in the *MDR1* promoter that have been implicated in the regulation of the *MDR1* gene were examined in the K562 and MDR cell lines by EMSAs. From this it was concluded that the transcription factor NF-Y binds to the *MDR1* Y-box, however nuclear levels of NF-Y do not vary between drug sensitive and drug resistant cells and do not change upon induction of MDR through drug exposure. The same conclusion was drawn for the protein binding to the –55GC box and the protein complex binding to the –120 to –112 NRE.

DNA methylation has long been implicated in the transcriptional regulation of many genes so bisulphite genomic sequencing was used to examine the methylation status of

the *MDR1* promoter in the K562 and K/EPR drug resistant cell lines. All CpG sites tested were unmethylated in the K/EPR cells and only CpG sites 43 and 44 were methylated in K562 which a scan for potential transcription factor binding sites revealed no apparently significant binding sites were present in this region. From this it can be concluded that demethylation of these sites is unlikely to play a role in the activation of *MDR1* gene.

Finally the half life of *MDR1* mRNA was determined for the K/EPR cells before and after drug treatment with epirubicin that induced both MDR and *MDR1* mRNA levels in the cells. The half life was determined to be 12.5 hours for both treated and untreated cells thus it can be concluded that increased *MDR1* mRNA levels found in cells after drug induction are not due to post translation mRNA stabilisation.

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